The use of tissue culture techniques to study pathogenicity of and select for resistance to three major diseases (E. Carotovora subspp., Pythium violae, Erysiphe heraclei) of carrot (Daucus carota L)

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THE USE OF TISSUE CULTURE TECHNIQUES TO STUDY PATHOGENICITY OF AND SELECT FOR RESISTANCE TO THREE MAJOR DISEASES (*E. carotovora* subspp., *Pythium violae*, *Erysiphe heraclei*) OF CARROT (*Daucus carota* L.)

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ABSTRACT

Highly embryogenic callus and suspension cultures of carrot (*Daucus carota* L.) were developed in order to generate, *via* somaclonal variation, disease resistance to three major pathogens of carrot in Europe for which there are no commercially used resistant cultivars i.e. *Erwinia carotovora* spp. (soft rot), *Pythium violae* (cavity spot) and *Erysiphe heraclei* (powdery mildew). Twenty seven percent of the regenerant seedlings screened from callus cultures expressed resistance to *E. heraclei*. Regenerant Somaclone-4 was significantly more resistant to powdery mildew than the original seed line NRI-92. However, this frequency of resistance among the regenerants was much higher than expected from somaclonal variation. It was concluded that variation may have come from the original seed used, because a high percentage of plants (ca. 40%) in the original seed line was free from powdery mildew. Nevertheless, it is clear that effective resistance already exists within the species.

Scanning electron microscopy revealed no differences in terms of *E. heraclei* infection structures on leaf surfaces of resistant Somaclone-4 and susceptible NRI-92; resistance must be expressed after penetration.

Six percent of roots of regenerants from embryogenic suspension cultures was resistant to *P. violae* and also the proportion of inoculated sites infected with *P. violae* was less than the original seed line cv. Morot Duke of which all roots developed cavity spot. Three percent of *E. carotovora* inoculated roots of regenerants was free from soft rot, in contrast to 18% of roots of cv. Morot Duke which remained symptomless after inoculation. These findings suggest that taproots showing resistance to *E. carotovora* may have arisen by one or a combination of: variation induced by tissue culture;

variation resulting from segregation in the seed line used; "escapes" resulting from an imperfect inoculation procedure. Reinoculation of cloned putative resistant individuals is required to confirm these possibilities.

Co-culture of carrot cells with both *E. carotovora* wild type and mutants and with *P. violae* was attempted with the aims of i) investigating determinants of pathogenicity and ii) regenerating putative, novel resistant plants from surviving embryogenic cells. Mycelial fragments of *P. violae* killed carrot cells very rapidly (< 24 hrs) such that this did not provide a suitable selection procedure. The pathogenicity determinants of *P. violae* are unknown, but cellulase, suberinase and pectin lyase were the major cell wall degrading enzymes, which may aid in penetration of the suberinized endodermis which surrounds carrot taproots. Carrot cells were killed rapidly (ca. 70% of the cell units and 80% of single cells in 24 hrs) with wild type *E. carotovora* (SCRI 193). Results *in vitro* and *in vivo* implied that the major pathogenicity factor of *E. carotovora* involved in maceration and killing of carrot cells was the extracellular cell wall degrading enzyme, pectate lyase (PGL). Thus pectate lyase killed carrot cells, enzyme deficient mutants (RJP 116 and RJP 243) had reduced pathogenicity and killed plant cells more slowly and to lower levels than wild type SCRI 193. However, in some cases cell killing in co-culture was initiated just before appearance of PGL, suggesting the involvement of another factor. The possible value of enzyme deficient mutants in screening carrot cells for disease resistance *in vitro* is discussed.

Kanamycin at 25 μg/ml was used successfully for removing bacteria from co-culture whilst retaining viability of embryogenic cell units; however the antibiotic markedly reduced their regeneration capacity. No mature somatic embryo was regenerated from co-culture. However, it should be possible with a more suitable antibiotic to select and regenerate plants from this developed co-culture technique.

CONTENTS

ABBREVIATIONS

ABA	abscisic acid
BAP	6-benzylaminopurine
ca.	circa
cel	cellulase
cfu	colony forming units
CMA	corn meal agar
CMC	carboxymethyl cellulose
cv.	cultivar
CVS	cell volume after sedimentation
CWDE	cell wall degrading enzymes
DDW	double distilled water
E. coli	*Esherichia coli*
E. heraclei	*Erysiphe heraclei*
Eca	*Erwinia carotovora* subsp. *atroseptica*
Ecc	*Erwinia carotovora* subsp. *carotovora*
Ech	*Erwinia chrysanthemi*
endo-PG	endo-polygalacturonase
exo-PG	exo-poly-α-D-galacturonosidase
FDA	fluorescein diacetate
GA	gibberellic acid
HR	hypersensitivity response
HSL	N-3-(oxohexanoxyl)-L-homoserine lactone
IAA	indole acetic acid
IEF	isoelectro focusing
KIN	kinetin
LB	luria broth
LS	Lin and Staba medium
MES	2-(*N*-morpholine)-ethanesulphonic acid
Mot$^-$	non motile mutant

MS	Murashige and Skoog medium
MS2	Murashige and Skoog medium with 20 g/l sucrose
NA	nutrient agar
NAA	1-naphthaleneacetic acid
NaPP	sodium polypectate
NRI	new red intermediate
OD	optical density
Out^+	secretory mutant
Out^-	non secretory mutant
PCV	packed cell volume
PEG	polyethylene glycol
pel	pectate lyase isozyme
PGL	pectate lyase
pI	isoelectric point
PL	pectin lyase
PME	pectin methylesterase
PMG	polymethylgalacturonase
Prt	protease
pv.	pathovar
PVP	polyvinylpyrrolidone
Rexl	regulatory mutant
RH	relative humidity
rpm	revolutions per minute
RVU	relative viscometric units
SDW	sterile distilled water
SEM	scanning electron microscopy
TEM	transmission electron microscopy
Tox^-	non toxic secretory mutant
V8	V8 juice agar
WA	water agar
Z	zeatin
µE	micro Einstein
µl	microlitre
1/10 th MS	10 times diluted Murashige and Skoog medium
2,4-D	2,4-dichlorophenoxyacetic acid
2-iP	isopentenyladenine

CHAPTER 1

GENERAL INTRODUCTION

1. CARROT

1.1. ORIGIN, DISTRIBUTION, DESCRIPTION AND TAXONOMY

The carrot *Daucus carota* L., originated from the anthocyanin-containing forms of *Daucus carota* ssp. *carota,* found in Afghanistan and was then introduced to Europe, Mediterranean, Tropical Africa, Australia, New Zealand and America by the sixteenth and seventeenth centuries (Ammirato, 1986).

The species, *D. carota* L., to which all cultivated and wild carrots belong, is a member of the Umbelliferae. It is a dicotyledon and a diploid with a low chromosome number (2n=2x=18) (Simon, 1985).

Carrot cultivation requires a temperature of 15-20 ^{o}C and can be planted in the spring, summer and autumn in temperate climates. Although annual forms are known, carrot is grown as a biennial crop (Simon, 1985).

Carrot taproots contain 88.3% water, 8.9% carbohydrates, 0.8% protein, 0.4% fat, 0.8% fibre and 0.8% ash (Purseglove, 1968) and are used as vegetables. The taproots are also a valuable source of vitamin A obtained from carotene and some cultivars have fairly important quantities of vitamins B1, C and E. Carrot seeds are also produced for an oil which is used for flavouring and in perfumery (Pawlicki and Sangwan, 1993).

1.2. ECONOMIC IMPORTANCE OF CARROT

The annual carrot production in the world has increased steadily since the early 1960s. During the 15-year period from 1979 to 1994, the world production rose by about 39% from 10.202 million mt (metric ton) to 14.176 million mt i.e. an average annual increase of about 2.6% (Table 1.1). Table 1.2 shows the main carrot producing countries in order of their production. China leads with nearly 2.5 million mt representing 17.5% of the world total followed by USA with 1.4 million mt or 9.7% of the world total (FAO Year Book, 1994).

Carrot is produced as the second most important field vegetable in the United Kingdom. In 1992-93 the production was about 45.8 mt per hectare.

Table 1.1: World carrot production based on FAO Year Book 1994

Region	Area harvested (×1000 ha)		Yield (kg/ha)		Production (×1000 mt)	
	1979-81	1994	1979-81	1994	1979-81	1994
World	511	666	19958	21280	10202	14176
Africa	51	66	10466	11147	533	739
N. C. America	46	64	28588	30766	1328	1958
South America	27	40	17496	17250	465	685
Asia	118	179	18958	22796	2230	4075
Europe	129	143	27921	31694	3591	4535
Oceania	5	6	29192	36246	141	229

mt: Metric ton **ha:** hectare **kg:** kilogram **N.C.:** North and Central

Table 1.2: Leading countries for world production of carrot based on FAO Year Book 1994

Country	Production (×1000 mt)
China	2475 F
USA	1384
Russian Federation	1250 F
Poland	786
United Kingdom	769
Japan	720 F
France	604
Italy	470
Netherlands	461 F
Ukraine	327
Canada	305 F
Spain	300 F
Germany	245
Mexico	236 F
Argentina	221 F
Indonesia	215 F
Turkey	200

F: FAO estimated

2. SOMATIC EMBRYOGENESIS IN CARROT

Somatic embryogenesis is the production of embryo-like structures from somatic cells. Williams and Maheswaran (1986) described this as the process by which somatic cells develop into plants through the characteristic embryological stages without fusion of gametes.

Somatic embryogenesis was first recognised by Steward and co-workers (1958) with carrot suspension cultures and Reinert (1959) with carrot callus cultures on semi-solid media. Since then, investigations of somatic embryogenesis in carrot cultures, both of the cultivated and wild varieties, have been widespread and it has become one of the model systems for research into plant regeneration. The process of somatic embryogenesis can be divided into four different stages: initiation, proliferation, maturation and germination.

Production of carrot somatic embryos from cell and tissue cultures occurs either directly or indirectly. The most common system in use involves the indirect mode of embryogenesis in which a disorganised growth of cells is first initiated in the presence of auxin (usually 2,4-D), forming a callus and probably initiating pro-embryo formation; further development of the embryos takes place after removal of the auxin from the culture medium (Nomura and Komamine, 1985; Zimmerman, 1993). The direct production of globular embryos from epidermal cells of carrot hypocotyl explants in the presence of 2,4-D, without the initial formation of callus tissue, was recently demonstrated by Masuda et al. (1995).

A large proportion of the cells in carrot suspension cultures are apparently toti-potent, but somatic embryos most probably derive from single cells near the surfaces of cell aggregates (Haccius, 1978; McWilliam et al., 1974). Those cell aggregates which are able to form embryos have been termed proembryogenic masses by Halperin (1966) and they usually have a highly nodular surface. The proembryogenic cells that make up the aggregates are small (*ca.* 20 μm diam.), densely cytoplasmic with small vacuoles and large starch grains and a relatively large nucleus. If the surface of the cell aggregate is wet, translucent and contains highly vacuolated and elongated cells, the cell aggre-gate is either non-embryogenic or contains non-embryogenic tissues (McWilliam et al., 1974; Nomura and Komamine, 1985; Smith and Street, 1974; Steward et al., 1964).

In carrot, somatic embryos generally show the same developmental sequence as zygotic embryos, developing through globular, heart, torpedo and cotyledonary stages. In early studies, somatic embryogenesis was considered to resemble zygotic embryogenesis, but somatic embryos, unlike zygotic embryos, had shorter suspensors (McWilliam et al., 1974). Distinct structural differences between somatic and zygotic embryos were also observed, with the structure of the former (e.g. the degree of

vacuolation, the plastid composition and the lipid body content of the embryo cells) being more similar to germinated zygotic embryos than to zygotic embryos themselves (Timmers, 1993).

Initially, it was thought that the explanted tissue on the primary auxin-containing medium underwent cellular dedifferentiation to produce unorganised cells and cell clusters and that transfer of these cells to secondary auxin-free medium initiated embryogenic development (Steward et al., 1964; 1968). Later work, however, suggested that embryo initiation possibly occurred during the primary culture stage and that the presence of auxin in the medium blocked their maturation (Halperin, 1966). Nomura and Komamine (1985) and Komamine and co-workers (1992) agreed that exogenous auxins were required for induction of embryogenic cell units from single cells in the first phase and that subsequent formation of somatic embryos was certainly inhibited by the presence of the auxin. The result is that the somatic embryos are rapidly initiated from the exterior cells of the proembryogenic masses, when they are transferred into auxin-free medium. Guzzo et al. (1994) studied the origin of totipotent cells in hypocotyl explants of carrot showing that proembryogenic cell aggregates arose from asymmetric multiplication of provascular tissue in the presence of auxin whereas the epidermis and parenchyma cells enlarged but did not proliferate. Therefore, epidermis and parenchyma cells were not considered to be involved in the formation of the embryogenic cells.

It has been shown that embryogenic and non-embryogenic cell cultures can be distinguished by the use of molecular markers (Pennell et al., 1992 and Sterk et al., 1991). One of these is the EP2 which is expressed in the protoderm of 60-celled globular embryos, the shoot apex of seedlings, developing flowers and maturing seeds of carrot (Sterk et al., 1991). Embryogenic cultures have also been recognised by using

monoclonal antibodies and a positive correlation was found between presence of embryogenic cells and the response to the monoclonal antibody JIM8 in carrot suspension cell cultures. It was demonstrated that the mainly embryogenic single cells described above could be recognised by this technique (Pennell et al., 1992).

The examination of IAA metabolism in embryogenic and non-embryogenic cells of carrot has shown that embryogenic cells contain fifteen times higher amounts of IAA than non-embryogenic cells. In carrot embryogenic cells, IAA was converted to IAAsp (indole-3-acetylaspartic acid) while in non-embryogenic carrot cells IAA was metabolised to oxIAAsp (oxindole-3-acetylaspartic acid) (Sasaki et al., 1994). Recent work by Kreuger and van Holst (1993) demonstrated that arabinogalactan proteins isolated from carrot seeds promoted development of proembryogenic somatic embryos from a carrot cell line which had lost its embryogenic potential when added at a concentration of 10 mg/l together with a low concentration 2,4-D into the embryo induction medium.

The induction of somatic embryogenesis is considered to be one of the most important *in vitro* techniques, since it is often assumed that the somatic embryos arise from single cells, producing bipolar structures which develop into genetically uniform plants on transfer to soil (Vasil, 1995). These are features which would be particularly significant if embryogenic systems are to be used for genetic transformation, *in vitro* selection or mass propagation, since it is less likely that either chimeric or genetically variable plants would be produced. These assumptions, however, are based on a limited amount of evidence from a small number of species and it is important that they should be confirmed on a case by case basis. In cassava, for example, transformation studies support the view that somatic embryos can arise both from single cells and from cell groups, according to the embryogenic system that is employed (Taylor et al., 1996).

2.1. FACTORS INFLUENCING SOMATIC EMBRYOGENESIS

The process of somatic embryogenesis is affected by many factors either singly or in combination. These include: genotype and explant; composition of culture medium (formulation and state of medium, sources and level of nitrogen, carbohydrates and growth regulators); environmental factors (light, culture vessels, gaseous composition and cell density).

Carrot plants have proved to be particularly useful in tissue culture studies. Many parts or pieces of the healthy and vigorous carrot plant excised at any time of development are, for example, able to produce somatic embryos in a suitable culture system. These may be taproots (Steward et al., 1958; Sussex and Frei, 1968), immature embryos (Steward et al., 1964), hypocotyls (Fujimura and Komamine, 1979a, b; Kreuger and van Holst; 1993), seedling roots (Smith and Street, 1974), petioles (Halperin, 1966; Schäfer et al., 1985) and protoplasts (Dudits, 1984; Ohyama et al., 1972). Within a species, however, different genotypes can show varying potentialities for somatic embryogenesis and Schäfer and co-workers (1985) reported that the embryogenic responses of three different carrot cultivars were different; one did not produce any embryogenic structure, one was highly embryogenic and the third only produced adventitious roots.

Somatic embryogenesis of carrot can be induced successfully with both semi-solid and liquid media (Steward et al., 1958; Reinert, 1959). Although many different types of basal media have been used for somatic embryogenesis of carrot, Evans et al. (1981) noted that the MS basal salt and vitamin formulation is the one most commonly used for the induction of somatic embryogenesis in crop plants, with 70% being cultured on MS medium or a modification of it.

It has been shown by a number of workers that a substantial amount of reduced nitrogen, such as ammonium ions and certain amino acids or amides together with nitrate in a medium, are necessary for both embryo initiation (Halperin, 1966; Halperin and Wetherell, 1965; Kamada and Harada, 1979) and maturation (Ammirato and Steward, 1971). Addition of certain nitrogen compounds such as alanine, glutamine, glutamic acid, urea, asparagine and aspartic acid were found to be beneficial when nitrate was also present in media (Kamada and Harada, 1979; Wetherell and Dougall, 1976). In contrast, other compounds such as leucine, isoleucine, lycine and histidine inhibit somatic embryogenesis in carrot (Kamada and Harada, 1984) and Ronchi and co-workers (1984) showed that proline and serine, when added to culture media during the growth of carrot suspensions, significantly stimulated the growth of embryos, but induced highly abnormal structures.

In 1977, Verma and Dougall pointed out that although many other mono and disaccharides could be employed successfully for initiation and development of carrot somatic embryos, sucrose appeared to be the most effective carbon source. Increased sucrose and addition of hexitols, such as myo-inositol or sorbitol, prevented precocious germination but increased secondary embryo formation (Ammirato, 1985; Ammirato and Steward, 1971). It has been argued that by increasing the osmotic concentration of the medium, plasmolysis is increased and the frequency of regeneration and the degree of synchronous development in suspension cultures of the wild carrot is enhanced (Wetherell, 1984).

As discussed above, somatic embryos generally arise as a result of the presence of an exogenous plant regulator. Many auxins can initiate the development of an embryogenic structure in carrot, e.g., IAA (Kato and Takeuchi, 1963; Sussex and Frei,

1968) and NAA (Ammirato, 1985; Ammirato and Steward, 1971) and 2,4-D (Ammirato 1985; Fujimura and Komamine, 1980a; Sasaki et al., 1994).

The effects of 2,4-D on the induction and development of somatic embryogenesis have been studied extensively. Many researchers have demonstrated that the presence of the auxin in the culture medium is necessary for the induction and proliferation of cells, and that it inhibits the subsequent development of somatic embryos from embryogenic cell units. (Halperin, 1966; Halperin and Wetherell, 1964; Fujimura and Komamine, 1980a; Komamine et al., 1992). The mode of the inhibitory action of auxins was examined by Fujimura and Komamine (1980a, b) and they showed that 2,4-D in the embryo-inducing medium completely blocked the multiplication of embryogenic cells and therefore somatic embryo development when it was placed into the medium in the first 3 days of culture. Thereafter, embryogenic cells devided very rapidly and the shoot, root and non-dividing regions were determined by the fourth day of culture. Michalczuk et al. (1992) showed a correlation between the embryogenic competence of cell lines and their 2,4-D contents during embryogenesis. Embryogenic lines, after being subcultured to 2,4-D-free medium, displayed a very fast reduction in both free and conjugated 2,4-D metabolites within seven days but the non-embryogenic line maintained the 2,4-D. It was also noticed that non-embryogenic cell lines contained higher levels of IAA than embryogenic lines. Antiauxins such as 2,4,6-trichlorophenoxyacetic acid decreased the elongation of somatic embryos and prevented the formation of root hairs but stimulated the aerobic respiration (Newcomb and Wetherell, 1970).

Carrot somatic embryos will mature completely in a medium free of cytokinins but Fujimura and Komamine (1980a) demonstrated that cytokinins will promote carrot somatic embryo initiation and they will also inhibit malformation of cotyledons

(Ammirato and Steward, 1971). In one of their studies, Fujimura and Komamine (1975) showed that zeatin (Z) but not kinetin (KIN) promoted somatic embryogenesis in single cell cultures of carrot. The role of Z was found to be very important in promoting embryogenesis in the 3 and 4 days of culture which was the active cell division stage. Z also supported maturation in low density caraway cultures and was most effective in combination with abscisic acid (ABA); Z and gibberellic acid (GA) stimulated growth but increased the frequency of aberrant forms (Ammirato, 1977). In contrast, ABA suppressed abnormal proliferation and fostered normal embryo maturation in caraway and carrot cultures (Ammirato, 1985; Kamada and Harada, 1981). The cytokinin, isopentenyladenine (2-iP) supported the initiation of embryo formation and also accelerated embryo development of 5-methyltryptophan resistant cell lines of wild carrot (Sung et al., 1979).

The presence of activated charcoal in the medium also promotes embryo development. Suspension cultures of carrot which failed to produce somatic embryos in hormone-free medium, regained this ability when activated charcoal was added (Drew, 1979; Fridborg and Eriksson, 1975). Charcoal probably assists differentiation of cells by adsorbing toxic substances and growth regulators that inhibit somatic embryogenesis when added to carrot, *Allium* and *Haplopoppus* cultures (Fridborg et al., 1978).

Different light conditions ranging from darkness to high levels and various lengths of photoperiod were all shown to be suitable for somatic embryo production from carrot (Ammirato, 1986) although it had previously been shown that a higher proportion of normal carrot somatic embryos was produced in complete darkness (Ammirato and Steward, 1971).

The types of culture vessels and oxygen level in the medium are also important for somatic embryogenesis of carrot. Ammirato (1986) noted that the type of vessels

could affect the frequency of aberrant embryos, but such differences in the maturation pattern might be a result of differences in the aeration of medium or in the amount of the contact with the medium. Kessel et al. (1977) showed that higher levels of dissolved oxygen favoured callus and root development, while lower levels promoted embryogenesis. One of the critical variables is population density and Halperin (1967) indicated that the density of carrot embryogenic cells in suspension affected the degree of embryo maturation, but it is not clear whether this was a result of differing oxygen concentrations. It has also been shown that globular proembryos developed normal embryos at high inoculum density (*ca.* 200 embryos/ml) whereas the proembryos simply enlarged without forming normal embryos at low inoculum density (*ca.* 60 embryos/ml) (Warren and Fowler, 1981).

3. SOME MAJOR CARROT DISEASES CAUSED BY FUNGI AND BACTERIA

Three major diseases of carrot, bacterial soft rot (*Erwinia carotovora*), cavity spot (*Pythium violae*) and powdery mildew (*Erysiphe heraclei*) for which there are no resistant cultivars in commercial use were studied in an attempt to obtain resistance using tissue culture techniques.

3.1. BACTERIAL SOFT ROT DISEASE CAUSED BY *Erwinia carotovora*

Bacterial soft rot in carrots caused by *Erwinia carotovora* subsp. *carotovora (Ecc)* (L.R. Jones) Holland and *Erwinia carotovora* subsp. *atroseptica (Eca)* (V. Hall) Jennison is one of the most destructive diseases in the field, in transit and in storage (Pérombelon, 1982; Pound, 1953; Romeiro et al., 1988). Examination of the etiology of soft rot on Peruvian carrots in Brazil revealed that *Erwinia carotovora* spp. were the

cause of the disease (Romeiro et al., 1988). Erwinias are gram-negative, motile, non-spore-forming facultative anaerobes and produce watery, smelly, soft rot of parenchymatous tissue, especially of storage tissues. Most strains produce large quantities of extracellular enzymes (Pérombelon, 1982; Pound, 1953).

The soft rot bacteria may survive in infected tissues and in the soil during winter and in contaminated equipment and containers. Some may also survive over winter in insects. These bacteria enter plants or plant tissues primarily through wounds or natural openings (stomata, hydathodes, lenticels) which is presumably the mechanism of infection of uninjured tissues. Under optimal conditions colonisation can be very rapid, such that a potato tuber could be completely rotted within 3 to 5 days (Kelman, 1979; Pérombelon, 1982; Pérombelon and Kelman, 1980).

It has been demonstrated that there are distinct differences in the aggressiveness of the two strains on carrot. *Ecc* was found to produce more severe tissue damage compared to *Eca* (Michalik et al., 1992). Coincidentally, growth rate of *Eca* was lower *in vitro* than that of *Ecc* (Pérombelon and Kelman, 1980; Wells, 1974).

Several characteristics of the natural environment influence the pathogenesis of soft rot bacteria. These factors include: oxygen (O_2), temperature, factors derived from plant tissues which stimulate pectate lyase (PGL) production, construction of plant cell walls and free water on the plant surfaces (Lyon, 1989; Pérombelon and Kelman, 1980).

Decreasing O_2 concentration causes a considerable increase in the amount of rotting, presumably by reducing the defence mechanisms of plant tissue, as it does not affect production of pectic enzymes by the pathogen (Leach, 1930; Maher and Kelman, 1983). It was also observed that optimal growth of *Ecc* occurred *in vitro* under aerobic rather than anaerobic conditions. In contrast, growth *in vivo* was more rapid when oxygen was limited (Maher and Kelman, 1983) partly reflecting by definition that

E. carotovora is a facultative anaerobe (Wells, 1974). Recently, Allefs et al. (1995) working on the resistance to *Eca* in backross (BC) populations of somatic hybrids between *Solanum tuberosum* and *S. brevidens* reported that the BC1 clones screened under aerobic conditions were more resistant than the parent used for BC1 production although no segregation among BC1 clones was apparent when tested under anaerobic conditions. These results suggest that an oxygen-dependent resistance mechanism is important for resistance to soft-rot erwinias.

The population of *Eca* on the surface of potatoes during storage was investigated by van Vuurde and Vries (1994) who found that intact surface, aeration of peeled surface of the tubers and dry soil reduced the presence of the *Eca* significantly.

Eca is generally effective on potatoes in cool climates. However, *Ecc* has a wide distribution range in both temperate and tropical regions and is able to attack many more crops than *Eca* (Pérombelon and Kelman, 1980).

Lyon et al. (1989) reported that if potato tubers inoculated with *Eca* were first incubated under aerobic conditions at 20 °C for 72h to initiate rotting and further for 72h at 10 °C, resistance responses of the tuber developed, preventing further rotting and bacterial multiplication. Temperature differentially affected the secretion of PGL by soft rot bacteria. PGL production by *Eca* was high at 15 °C but undetectably low at 30 °C whereas enzyme production by *Ecc* was equally high at both temperatures (Pérombelon et al., 1979).

Pérombelon et al. (1979) noticed that when *Eca* and *Ecc* were inoculated into tubers and incubated under certain conditions, *Eca* predominated at low temperatures (*ca.* 16 °C) and *Ecc* at high temperatures (*ca.* 26 °C). However, at 22 °C both bacteria grew in tubers at equal rates. High temperatures (above 20 °C) and high humidity increase the rotting of the tubers. This is supported by Segal and Dow (1973) who

illustrated that three days storage at 2 °C followed by 4 days at 21 °C reduced the amount of rotting caused by *E. carotovora* more than in carrots held for 4 days at 21 °C. Holding carrot slices at low temperature (e.g. 2 °C) for at least 2 or more days greatly reduced the incidence of decay. They concluded that when carrot roots were kept at 2 °C phenolic or related compounds may be formed in the roots.

Phytoalexins, low molecular-weight, induced antimicrobial compounds are known as factors inhibiting the growth of some pathogens. They are produced in plants only after stimulation by micro-organisms or chemical and mechanical injury (Lyon, 1989; 1992). Accumulation of phytoalexins such as rishitin was found to be dependent on the temperature. A high number of *Eca* was killed by a high concentration of rishitin (500 μg/ml) when they were incubated at 30 °C. There were no viable bacterial cells at 30 °C compared with 8.6 10^7 cfu/ml at 10 °C (Lyon, 1984). Rapid and extensive maceration of potato tubers incubated under anaerobic conditions was correlated with the absence of phytoalexin accumulation while tubers incubated under aerobic conditions accumulated considerable amounts of rishitin and smaller amounts of solavetivone and phytuberin and underwent less extensive rotting (Hildenbrand and Ninnemann, 1994).

One of the important factors which can influence the susceptibility of plants to *E. carotovora* is the mineral nutrition supplied to plants. McGuire and Kelman (1984) showed that increased calcium content in potato tubers was paralleled with resistance to *Eca* perhaps because a high amount of calcium enhances the structural integrity of plant cell walls and membranes. Also, bacterial stem rot (*Ecc)* on hydroponically grown tomatoes was suppressed with a high potassium/nitrogen ratio in the nutrient solution (Dhanvantari and Papadopoulos, 1995).

3.1.1. Pathogenesis by Soft Rot *Erwinias*

In this study attempts are made to determine possible pathogenicity factors of *E. carotovora.* Pathogenicity factors might be revealed by using either co-culture with host cells or by defined mutants and could also be exploited in somaclonal variation. In addition, mutants lacking a toxic factor may be more suitable than wild type as a selection agent in somaclonal variation. Two obvious effects on host tissues and cells are rapid cell maceration in infected carrot tissues and rapid cell killing *in planta* and in co-culture of plant cells by *E. carotovora.* From previous studies the three main types of pathogenicity factors of soft rot *Erwinia* might be associated with (a) extracellular enzymes in particular those which degrade plant cell walls; (b) the cell surface of the pathogen and (c) unknown factors coded for by genes induced in the presence of host plant extracts (Salmond, 1994).

Most evidence implicates certain cell wall degrading enzymes in soft rot diseases of plants caused by *E. carotovora* (Basham and Bateman, 1975a, b; Collmer and Keen, 1986; Cooper, 1983; Ried and Collmer, 1986; Salmond, 1994; Stephens and Wood, 1975; Tanebe et al., 1987). Therefore, cell wall degrading enzymes as possible determinants of pathogenicity of soft rot bacteria were assessed.

3.1.1.1. Cell Wall Degrading Enzymes

Most research implicates extracellular cell wall degrading enzymes (CWDE) capable of cleaving the major glycosidic linkages in plant cell walls of the host tissue, in pathogenicity of erwinias (Cooper, 1983; 1984). These CWDE comprise pectinases, cellulases, hemicellulases and proteases (Bateman and Basham, 1975; Collmer and Keen, 1986; Kotoujansky, 1987).

Pectic substances make up the major component of the middle lamella and a portion of the primary plant cell wall, so the corresponding CWDE can play an important role in the determination of pathogenicity in soft rot erwinias (Bateman and Basham, 1975; Cooper, 1983; Stephens and Wood, 1975).

The pectic polymer, rhamnogalacturonan, is degraded by two different enzymatic mechanisms; hydrolysis (by endo-polygalacturonases [endo-PG] and exo-poly-α-D-galacturonosidase [exo-PG]) and ß elimination (by lyases e.g. endo-pectate lyase [endo-PGL], pectin lyase [PL] and exo-pectate lyase [exo-PL]), which are produced in abundance by all members of the soft rot erwinias. The hydrolases and lyases are distinguished by their differing pH optima (*ca.* 4-5 for PGs versus *ca.* 8-10 for lyases), by their requirement for Ca^{+2} (lyases have a requirement for Ca^{+2} unlike the PGs) and by their products (only lyases result in an unsaturated bond between carbon 4 and 5 at the non-reducing end of the broken chain). Both may attack internal regions of chains at random (endo-) or terminally (exo-) but some combine the two modes of action and attack chains at random, release mono-or oligomers (multiple attack). The methoxy groups on pectic acids are removed by pectin methylesterase (PME) which can therefore facilitate the action of depolymerases specific for free carboxyls (Bateman and Basham, 1975; Collmer et al., 1982; Cooper, 1983).

Many investigations have revealed that the predominant pectic enzyme secreted by soft rot *Erwinia* spp. is PGL; the unique damaging effects of endo-pectinases on plant tissues has been demonstrated by many researchers (Basham and Bateman, 1975a, b; Bateman and Basham, 1975; Cooper, 1983; Kotoujansky, 1987; Mount et al., 1970; Ried and Collmer, 1986). Purified endo-PL and endo-PGL obtained from *Ecc, Eca and E. chrysanthemi (Ech)* cause maceration, electrolyte loss, permeability changes and cell killing of the plant tissues (Basham and Bateman, 1975a, b; Collmer and Keen, 1986;

Garibaldi and Bateman, 1971; Mount et al., 1970; Ried and Collmer, 1986; Willis et al., 1987). The evidence of Basham and Bateman (1975b) led them to conclude that endo-cleaving pectic enzymes killed plant cells by rendering them osmotically fragile. Plant tissues treated with pectic enzymes show rapid and irreversible increase in permeability as a result of plasmalemma damage. Plasmolysis of plant tissues inhibits the toxic effect of pectic enzymes. This toxic effect on plant cells is dependent on whether cells are plasmolyzed or deplasmolyzed. In deplasmolyzed cells the plasmalemma is held tightly against the cell wall by turgor pressure; following loss of wall integrity by PGL action. Splits and tears in the cell membrane may occur and this is followed by death of the plant cells (Bateman and Basham, 1975; Kelman, 1979). The viability and permeability changes of plant cells were measured by vital stain and leakage of electrolytes from infected tissues respectively (Bateman and Basham, 1975). Rapid cell killing was also observed when apple cells were cultured with PL from *Monilinia fructigena*, but plasmalemma damage occurred almost immediately and before detectable cell wall breakdown, implying that simple osmotic damage was not the mechanism of killing (Keon, 1985). Yamazaki et al. (1983) reported that acid-released pectic cell-wall fragments inhibited leucine incorporation into protein in suspension cultured sycamore cells, unless the cells were plasmolyzed. In addition, Stephens and Wood (1975) showed that some toxic factor(s) other than PGL, killed protoplasts of plasmolysed tissues. Purified PGL killed unplasmolysed protoplasts but not the protoplasts of plasmolysed tissues demonstrating that toxicity is indirect and protoplasts need to be in intimate contact with their cell wall in order to be damaged by PGL.

Soft rot inducing erwinias have provided model systems to investigate pathogenesis by molecular genetic analysis (Chatterjee et al., 1995; Collmer et al., 1982; Heikinheimo, 1995; Jones et al., 1994; Kotoujansky, 1987; Lei et al., 1985a, b; Liu

et al., 1993; Mäe et al., 1995; Roberts et al., 1986; Salmond, 1994 ; Stack et al., 1980; Zink and Chatterjee, 1985). Evidence based on isoelectro focusing (IEF) has demonstrated that multiple isozymes of PGL, PL and PG are produced by *Ecc*, *Eca* and *Ech*. The isozymes of PGL are grouped to "alkaline", "neutral" and "acidic" with approximate isoelectric point (pI) values of 9.0 to 10.0, 7.0 to 8.5 and 4.0 to 5.0 respectively (Kotoujansky, 1987; Ried and Collmer, 1986). In early studies, three PGL isozymes (pelA, pelB and pelC) with isoelectric points higher than 8.7 and one PG with pI of *ca.* 10.2 were observed in *Ecc* and *Eca* (Lei et al., 1985b; Ried and Collmer, 1986). Later work on separation of pectolytic enzymes using IEF and quantitative assay showed that *Ecc* strain *Ecc*71 produced five PGLs (pI 10.0, 9.7, 9.2, 8.0 and 6.6), one endo-PG (pI 10.0) and one exo-PG, whereas *Eca* strain SR-8 produced six PGLs (pI 10.2, 9.6, 9.5, 9.4, 9.2 and 8.9), two endo-PGs (pI 10.7 and 3.9) and one exo-PGL (pI 7.1). Although all PGLs and endo-PGs were extracellular, two of six PGLs and one exo-PG were also found in the periplasm in *Eca.* However, two of the five PGLs were periplasmic and the rest were extracellular in *Ecc* (George et al., 1991; Hinton and Salmond, 1987; Willis et al., 1987). Stack et al. (1980) reported that one of the three intracellular PGLs was capable of producing dual enzymatic activity in *Ecc.* They showed that both exo-PGL and endo-PG had identical properties i.e. in reaction mixture at pH 8.5 containing Ca^{+2}, pectate was degraded in a lyitic manner, resulting in formation of unsaturated oligouronides. At pH 6.0 in the presence or absence of ions, cleavage of pectate was effected in a hydrolytic manner, only saturated products were produced. Godfrey and co-workers (1994) detected PL activity in rotting potato tuber tissue inoculated with *Eca.* It was also shown that purified PL from *Eca* was capable of macerating potato tuber tissues. To characterise the role of individual PGLs in virulence, targeted mutations were applied into the coding region of the pel genes and mutants

which are defective for only one PGL or for a combination of several PGL were obtained. The recent work by Heikinheimo et al. (1995) revealed a novel PGL secreted by another *E. carotovora* strain SCC3193. It was illustrated that one of four different PGL isoenzymes, pelB did not play a major role in the virulence of *Ecc* because a mutant lacking pelB gene macerated tobacco leaves, potato stems and tubers as well as the wild type. Possibly related results were found much earlier by Garibaldi and Bateman (1971) working on the effects of pectic enzymes produced by *E. chrysanthemi* on plant tissues. They reported that isoenzymes of PGL had a different effect on the maceration of various plant tissues. Carrots treated with isozyme II (pI 7.9-8.4) were resistant to maceration but not when treated with isozyme I (pI at pH 9.4). However, potato and cucumber tissues were quite susceptible to maceration by both isozyme types. Boccara et al. (1988) investigated whether all PGL isozymes of *Ech* were necessary for the virulence on whole *Saintpaulia ionantha* plants. They reported that: i) PelB, PelC and PelB PelC double mutants were pathogenic; ii) PelD, PelE, PelD PelE were avirulent; iii) PelA, reduced PGL mutant, was pathogenic but delayed the development of symptoms. In another work, Roeder and Collmer (1985) did not find any significant differences between wild-type and PelB, PelC mutants of *Ech* CUCPB 1237 in the maceration of potato tubers. Quantick et al. (1983) illustrated that different PGL isoenzymes contributed differently to the virulence of *Eca*. Despite the production of three PGL isoenzymes by *Eca in vitro*, only one was present in extracts of rotting potato tubers.

In order to characterise each pectic enzyme individually, researchers have used the strategy of cloning and expressing the genes in *Esherichia coli* , a non-pectic enzyme producing host. This is facilitated because *E. carotovora* is a member of the Enterobacteriaceae and is related to *E. coli* (Brenner et al., 1973). PGL encoding genes

(pel genes) from *Ecc*, *Eca* and *Ech* have been cloned in *E. coli* via plasmids or lambda phage (Allen et al., 1987; Kotoujansky, 1987; Lyon, 1984). Cloning of the genes encoding pectolytic enzymes allow us to determine the number of genes, their regulation and relationship to the pathogenic process of plant cell wall hydrolysis.

The role in pathogenicity of genes encoding PGLs and PG of *Ecc* were examined by Lei et al. (1985a). PG protein without any PGL contamination made in *E. coli* macerated plant tissue. PG was detected in the culture fluids of *E. coli*, whereas the PGLs were not secreted. PG encoded into *E. coli* cosmid and cell extracts of *E. coli* containing cosmid that encodes PG were capable of macerating carrot, potato or turnip slices. It was concluded that PG alone was able to macerate plant tissues.

Maceration of potato tuber tissue by *E. coli* clones harbouring pectinase genes from *Ecc* has also been reported but in that case, only a clone expressing simultaneously an endo-PL, an exo-PL and an endo-PG was able to cause limited maceration (Roberts et al., 1986).

Tanebe et al. (1987) examined the effect of endo-PL and endo-PGL obtained from *E. carotovora* on the enzymatic maceration of mitsumata (*Edgeworthia papyrifera* Sieb. et Zucc) bast. They found that endo-PGL was the dominant maceration factor with the supplementary aid of endo-PL.

Degradation of highly methylesterified pectin by endo-PL activity was shown by most soft erwinias, when induced with mitomycin C (Godfrey et al., 1994; Tsuyumu and Chatterjee, 1984). PL activity was generally higher in *Ecc* than in *Eca* and *Ech* (Tsuyumu and Chatterjee, 1984).

Reduced virulence mutants of *Eca* and *Ecc* were isolated from either chemical mutagenesis using ethylmethane sulphonate (EMS) or transposon mutagenesis *via* Tn*phoA*. It was shown that these mutants still synthesize pectinases and cellulases but

these enzymes accumulate within the periplasm (Hinton et al., 1989; Liu et al., 1993; Reeves et al., 1993).

Some of these mutants were employed in this study (Hinton and Salmond, 1987; Liu et al., 1993; Murata et al., 1990; Mulholland et al., 1993; Reeves et al., 1993; Ried and Collmer, 1986).

Yang et al. (1992) has developed a model for the role of pectic enzyme regulation in host-pathogen interaction. This model suggests that the dual functions of pectic enzymes in pathogenesis, cell wall degradation and elicitor synthesis, are mediated by the regulation of these enzymes. When the production of these enzymes is up-regulated, their function is shifted towards generation of elicitor of plant cell wall inactive oligogalacturonides, which can be further catabolized to produce inducers of pectic enzymes. When pectic enzymes are down-regulated, low levels of endo-PGL allow generation of elicitor active oligogalacturonides that induce plant defence responses. Regulation of exo-PGL and possibly of exo-PG is critical, particularly early in the interaction. Decreases in exo-PGL moved the interaction towards defence activation; increases in exo-PGL moved the interaction towards pathogenesis. Other factors including pH (<6) of plant surfaces and tissue or the pH (>8) of damaged cells may also affect enzyme activity.

In *Ecc, Eca* and *Ech*, several individual regulatory loci which are involved in the regulation of the pectinases e.g. pehR, pecS, pecI and kdgR and global regulatory loci which regulate production of all the extracellular enzymes e.g. Aep, Rex, Rsm and Exp have been detected (Cui et al., 1995; Liu et al., 1993; Murata et al., 1990; 1994; Pirhonen et al., 1991; 1993). The global regulatory system is affected by thermoregulation mechanism such that well below their maximal growth temperatures, *Erwinia* spp. will down-regulate the synthesis of the secreted enzymes (Salmond, 1994).

Mutations that affect down-regulation of the extracellular enzymes lead to a reduction or loss of virulence in planta while mutations that affect up-regulation lead to a hyperproduction of enzymes in planta (Murata et al., 1990). Transposon insertion Out$^-$ (non-secretory) mutants were isolated from *Ecc* 71 by using plasmid and bacteriophage lambda delivery systems. These mutants did not excrete pectate lyases, polygalacturonase, and cellulase into the medium, but they accumulated in the periplasm. The Out$^-$ mutants showed significantly reduced virulence on potato tuber tissue, whereas Out$^+$ (secretory) mutants regained the ability to macerate potato tuber tissue (Murata et al., 1990). Willis et al. (1987) reported that when either mutant that lost endo-PG activity or wild type were inoculated into carrot, radish, celery, or potato tissue, extensive maceration occurred in the inoculated areas under either an aerobic or an anaerobic environment. Therefore, they concluded that endo-PG contributed to disease development but did not reveal a detectable change in virulence.

Two types of reduced virulence mutants (Rvi$^-$) were obtained in *Eca*. These were mutants affected in enzyme production and secretion (Hinton et al., 1989) and mutants which were able to synthesize the exoenzymes but were Rvi$^-$ (Mulholland et al., 1993). The reduced virulence mutants of *Eca* were characterised by Mulholland and co-workers (1993). Two mutants were found to be non-motile because of the flagella structures. Flagellar assembly proteins and pathogenicity proteins were homologous and are involved in plant pathogenesis. These mutants showed pleiotrophic phenotypes and were simultaneously resistant to a set of *Eca* bacteriophages.

Transposon mutants of *Ecc* exhibiting either reduced virulence (non-motile mutant, Mot$^-$) or avirulence (exoenzyme-deficient mutants, Exp$^-$) phenotypes were tested to determine pathogenicity on tobacco seedlings (*Nicotiana tabacum)* and potato. The wild type *Ecc* strain produced extensive maceration in 2 days on tobacco seedlings

and spreading black lesions in the stem tissues of potato, while Mot$^-$ and Exp$^-$ mutants tended to produce either small, slowly spreading lesions or no symptoms. It was concluded that motility was an important virulence factor of *Ecc* for the rapid spread of bacteria inside the plant tissue (Pirhonen et al., 1991). Palva and co-workers (1993) revealed that non-motile mutants (Mot$^-$) secreted wild type levels of exoenzymes, seemed to multiply in plants and tended to produce small rotting lesions while avirulent exoenzyme deficient mutants (Exp$^-$) did not multiply at all in plants and also cause maceration.

E. carotovora mutants defective in synthesis of pectinase, protease and cellulase exoenzymes appeared to be completely avirulent, but exoenzyme production was restored by exogenous addition of N-3-(oxohexanoyl)-L-homoserine lactone (HSL) (Jones et al., 1993; Pirhonen et al., 1993). HSL was shown to regulate both carbapenem antibiotic production and exoenzyme synthesis in *E. carotovora* and restore elastase which is a virulence determinant in *P. aeruginosa* (Jones et al., 1993). A novel RsmA$^-$ (Rsm, repressor of secondary metabolites) mutant which overproduces extracellular enzymes was obtained recently by Chatterjee and co-workers (1995). RsmA$^-$ mutant was able to produce extracellular enzymes in the absence of HSL and also caused more extensive maceration of plant tissues than the parent RsmA$^+$. Cui and co-workers (1995) proved that a negative-global regulatory loci, rsmA, was responsible for suppression of extracellular enzyme production, HSL synthesis and pathogenicity in many soft-rotting *Erwinia* spp.

There has been little work on hemicellulose and cellulose degradation by *Ecc*. It has been shown that *Ecc* produces two different cellulases and one protease which may aid in the degradation of plant cell wall components and cytoplasmic membranes respectively (Mäe et al., 1995; Willis et al., 1987). Cellulase was claimed to be able to

macerate tobacco leaf tissues. Mäe et al. (1995) illustrated that mutants impaired in the major cellulase, Cel VI production, showed highly reduced virulence in plant tissues. Protease purified from *E. carotovora* subsp *carotovora* caused partial cell death on cucumber disks and lysis of cucumber protoplasts (Tseng and Mount, 1974).

3.2. CAVITY SPOT DISEASE CAUSED BY *Pythium violae*

The term cavity spot was first used by Guba et al. (1961) to describe symptoms consisting of watersoaked, sunken elliptical lesions on the roots of carrot (*Daucus carota* L.) and parsnip (*Pastinaca sativa* L.) grown in Massachusetts, USA. Microscopic studies of cavity spot lesions revealed that cavities first appeared underneath the epidermis and were formed by the initial collapse of tissue beneath the epidermis followed by rupture of the covering tissue. The lesions assumed the form of sunken cavities up to 3-4 mm deep and 0.2-4 mm in diameter (Guba et al., 1961). Perry and Harrison (1979a) gave a more detailed account of the disease symptoms. They revealed that the outer layer of cells of the phloem parenchyma aggregated and then collapsed. After that the cells of the periderm and pericycle disintegrated. A layer of wound periderm under the lesion was formed by meristematic activity and then the periderm tissue showed secondary thickening due to the deposition of lignin and production of suberin. Fungal hyphae and bacteria were observed in the lesions.

The cause of cavity spot was uncertain. Many factors were considered to affect the development of cavity spot on carrot roots. In 1961, it was thought that decreasing calcium (Ca^{+2}) levels in the host increased the incidence of cavity spot. Increased Ca^{+2} level was found to be related to reduction of the K^{+} in the plants and decrease of the cavity spot formation (Maynard et al., 1961; 1963). However, the results of field surveys and pot experiments carried out by Perry and Harrison (1979b) showed that neither Ca^{+2}

nor K^+ concentrations in the soil or their ratios were associated with cavity spot formation of carrots in East Scotland. They concluded that environmental conditions particularly lack of aeration and waterlogging were important for the initiation of symptoms. Similar results were also found by Hafidh and Kelly (1982) and Vivoda et al. (1991). They showed that the Ca^{+2} content of the roots declined as a result of elimination of Ca^{+2} from the nutrient solution but there was no correlation between Ca^{+2} reduction and incidence of cavity spot.

The incidence and severity of cavity spot was extensively reduced by inter-row cultivations both on narrow beds and in single rows (Perry, 1983). Anaerobic pectolytic spore forming bacteria belonging to the genus *Clostridium* were isolated and shown to be associated with cavity spot lesions. Perry and Harrison (1979b) demonstrated that *Clostridium* spp. lesions which resembled natural cavity spot found in the field occurred when carrot roots were grown in high soil water content pots exposed to temporary anaerobic conditions. It was also found that soluble carbohydrates were lost from carrot roots *in vitro* and the amount rose when anaerobic conditions were applied. They concluded that carrots might lose carbohydrates similarly in soil which could stimulate the anaerobic microflora under conditions of reduced oxygen availability.

Feeding injury by a fungus gnat larvae (*Bradysia impatiens*) was found in carrots growing in soils untreated with fungicide and caused cavity spot like lesions (Hafidh and Kelly, 1982). It was also found that the age of the carrot plants influenced the incidence of cavity spot. Five-month-old carrots inoculated with either *P. violae* or *P. ultimum* produced approximately twice as many lesions as 3-or 4-month-old carrot roots (Vivoda et al., 1991).

The soil-borne fungus, *Pythium violae* (Chester and Hickman) the causal agent of cavity spot of carrot, is a major economic problem limiting carrot production in UK,

Ontario, Norway, France, Israel, California and Netherlands (Guba et al., 1961; Liddell et al., 1989; Lyshol et al., 1984; van der Plaats-Niterink, 1975; Vivoda et al., 1991; White et al., 1993). In 1988, the high incidence of cavity spot was demonstrated in California where more than 10,000 ha. area was cultivated with carrot (Vivoda et al., 1991). Significant damage to carrot crops has been reported from some regions in the San Joaquin valley of California (Liddell et al., 1989), where infection of up to 80% of the carrot roots was observed. Cavity spot reduces the quality and makes carrot roots unsuitable for the fresh market and also causes rejection of crops for processing and pre-packing (Groom and Perry, 1985; Liddell et al., 1989). Identification of *Pythium* spp. by using serological techniques indicated that cavity spot was the main disease of 14,000 ha. of carrots grown annually in the UK seriously limiting crop production in areas of England and Scotland (Lyons and White, 1992).

A few *Pythium* spp. were isolated from cavity spot lesions, although the diverse genus *Pythium* comprises approximately 120 species (Martin, 1995). Several species of *Pythium* were shown to produce cavity spot. Slow-growing fungi *P. violae and P. sulcatum* have been the most commonly isolated (White, 1988; White et al., 1987, 1993). It was also shown that fast-growing fungi *P. intermedium, P. sylvaticum P. ultimum* and *P. irregulare* could be linked with cavity formation (Kalu et al., 1976; Liddell et al., 1989; Mildenhall, et al., 1971; Vivoda et al., 1991). However, many researchers agreed that *P. violae* was the primary and most widespread cause of cavity spot of carrots (Blok, 1987; Groom and Perry, 1985; Lyons and White, 1992; Montfort and Rouxel, 1988; Vivoda et al., 1991; White, 1986). Determination of the host range of *P. violae* in the greenhouse and in natural field soil revealed that cowpea, broccoli, celery, cucumber, sugar beet, watermelon and cauliflower were not hosts for *P. violae* but susceptible hosts to cavity spot were carrot, alfalfa and wheat (Schrandt et al., 1994).

Pythium violae was first isolated by Chesters and Hickman from varieties of Viola. In 1944 than described *Pythium violae* as different from other *Pythium* spp. with restricted occurrence of sporangia in host tissue and in culture and the abundant production of oospores. Mycelium consists of hyphae with a diameter of 3-4 μ and lacks septa except when cultures are old. Sporangia are produced at random over the mycelium and may be in a variety of shapes; spherical or subspherical, terminal or intercalary. Oogonia can be terminal or intercalary, smooth, spherical or subspherical and 25.7 μ in diameter. The oospores are also smooth and spherical with an average diameter of 20.6 μ. Two types of antheridia are produced by fungus. These are the slightly curved and barrel-shaped androgynous type and cylindrical, straight diclinous type.

White and co-workers (1993) reported that optimum temperature for growth of *P. violae* isolates from the UK, France and Israel was 20 °C or lower. Vivoda et al. (1991) also demonstrated that *P. violae* and *P. ultimum* were more virulent at 15 °C than 20 or 25 °C.

In order to evaluate pathogenicity of *Pythium* spp. many isolates must be tested since different isolates of *Pythium* spp. showed marked differences in virulence (White et al., 1987, White, 1988). The early report by Mildenhall and co-workers (1971) on *Pythium* brown root and forking of muck-grown carrot was important because they illustrated marked differences in disease incidence and severity between identical clones of the same species grown on the two different natural media PDB (potato-dextrose broth) and CE (corn extract).

Control of cavity spot caused by *P. violae* can be obtained by the use of fungicides such as metalaxyl, methyl bromide, propamocarb and fosetyl-Al, Fubol (Lyshol et al., 1984; White, 1986). White (1986) working on the effects of metalaxyl

and methyl bromide on cavity spot incidence, confirmed the findings of Lyshol et al. (1984) using metalaxyl and methyl bromide reduced the incidence of cavity spot. Perry (1983), Gladders and Crompton (1984) conducted field and pot experiments. They found that the severity and incidence of moderate cavity spot lesions were reduced, but metalaxyl did not diminish the number of minor lesions. White (1988) showed that treatment of carrots with mancozeb generally reduced the percentage of carrots with cavities and the mean number of cavities per root but it did not affect the size distribution of cavities.

Inoculation of 19 cultivars of carrot with three *Pythium* spp. (*P. violae, P. sulcatum, P. intermedium*) in order to screen for resistance revealed no useful cultivars resistant to cavity spot (White et al., 1987). Sweet et al. (1986) also failed to obtain cavity spot resistant carrot cultivars when they inoculated 51 carrot varieties with *P. violae*.

Although chemicals can be successfully used for control of cavity spot on carrots, ideally control should be achieved with resistant cultivars but there appear to be none currently available.

3.2.1. Pathogenesis by *Pythium* spp.

It is known that *Pythium* spp. penetrate most often directly through the unwounded surfaces of their hosts by infection pegs or slender infection hyphae. Some can also enter plant tissues through stomata and wounds (Chérif et al., 1991; Endo and Colt, 1974). The anatomy of the carrot root was reported by Esau (1940). She showed that the outer surface of the carrot root is surrounded by the periderm and a pericyle is found underneath the periderm which surrounds the phloem. Secondary xylem is found

at the centre of the root. Both secondary xylem and phloem tissues contain a large proportion of parenchymatous cells.

Generally all pathogens including *P. violae* encounter the cell wall barrier and must penetrate it if they are to achieve a successful infection. The periderm of carrot is a strong barrier and, if undamaged, can prevent the entry of most pathogens. It is thought that its suberized walls act as a physical barrier and accumulation of inhibitors like falcarindiol and 6-methoxymellein are capable of acting as biochemical barriers in the pericycle region (Davies and Lewis, 1981).

P. violae penetrates the outer suberized endodermis. This must presumably involve degradation of toughened cell walls impregnated with suberin. Suberin is an insoluble, hydrophobic polymeric material and is found preformed in periderm cell walls. In higher plants, cutin takes its place in the aerial parts, while suberin was found in the ground parts and at wound surfaces. Suberin contains both phenolic and aliphatic components. The phenolic matrix, which is similar to lignin, is attached to the cell wall and the covalently attached aliphatic components are linked to this core (Kolattukudy, 1980). Suberin is similar in composition to the aliphatic monomers of cutin, except that suberin contains phenolic groups. In contrast to cutinases, which have been extensively studied, little is known of suberinases; however, the structural similarity of suberin to cutin allows the use of similar enzyme assay procedures (Fernando, et al., 1984). Since *P. violae* is known to penetrate the periderm it may require the production of suberinase which is thought to be similar if not identical to cutinase in properties (Kolattukudy, 1980).

Investigations on the mechanisms by which *Pythium* spp. produce their disease effects have concentrated on the ability of *Pythium* spp. to secrete pectic and cellulolytic enzymes and phytotoxic fungal products. These cell wall degrading enzymes facilitate

cell wall penetration. Pectinases cause indirect host cell killing. No reports have been found concerning pectinolytic and celluloytic enzymes production by *P. violae* in contrast to production *in vitro* and *in vivo* by *P. aphanidermatum* (Deacon, 1979; Sadik et al., 1983; Winstead and McCombs, 1961), *P. ultimum* (Chérif et al., 1991; Deacon, 1979; Mellano et al., 1970), *P. debaryanum* (Deacon, 1979; Wood and Gupta, 1958), *P. nunn* (Elad et al., 1985), *P. butleri* (Deacon, 1979; Janardhanan and Husain, 1974), *P. sylvaticum* (Deacon, 1979; Nemec, 1974), *P. intermedium* (Deacon, 1979), *P. irregulare* (Deacon, 1979; Nemec, 1974), *P. perniciosum, P. dissotocum* (Nemec, 1974), *P. graminocola, P. mamillatum, P. scleroteichum* (Deacon, 1979). Pectic enzymes and other polysaccharide degrading enzymes have been reported to be able to degrade plant cell walls and macerate tissues in many plants (Cooper, 1983; Endo and Colt, 1974; Janardhanan and Husain, 1974; Sadik et al., 1983).

High endo-PG activity found in the crude culture filtrates of *P. ultimum* correlated with rapid maceration of susceptible snapdragon (*Antirrhinum majus*). Endo-PG activity declined when the host sterols such as β-sitosterol were used *in vitro*. However, attempts to correlate increases in β-sitosterol with increased tolerance of plants to the fungus failed (Mellano et al., 1970).

Cottony-leak disease of cucumber caused by *P. aphanidermatum* was found to be related to the activities of fungal pectinolytic and cellulolytic enzyme (Winstead and McCombs, 1961). A variety of pectinolytic enzymes were secreted by *P. butleri in vitro*. These were; endo-PG, endo-polymethylgalacturonase (endo-PMG), exo-PG and pectin lyase (PL). The fungus was also found to secrete weak cellulase but no pectin methyl-esterase (PME) (Janardhanan and Husain, 1974). PG and/or depolymerase activities were observed in culture filtrates when *P. aphanidermatum* was grown in potato broth medium but not in synthetic medium. No PME was detected, while high amounts of

cellulases were present in the fluids (Sadik et al., 1983). Nemec (1974) studied the *in vitro* production of pectinases and cellulases by six *Pythium* spp. isolated from necrotic strawberry roots. He showed that *P. irregulare, P. perniciosum, P. sylvaticum* and *P. dissotocum* which all had a limited ability to penetrate root, produced PG and PMG while none of them produced detectable pectin lyase. *P. sylvaticum* was the only species which secreted cellulase among the four species tested.

Deacon (1979) investigated 19 *Pythium* spp. in terms of their cellulase production and found that *P. graminicola, P. intermedium, P. irregulare, P. mamillatum, P. scleroteichum, P. sylvaticum* and *P. acanthicum* were highly cellulolytic while *P. anandrum, P. splendens, P. vexans, P. ultimum* and *P. butleri* were non-cellulolytic. Baker and Bateman (1978) demonstrated cutinase activity in the culture fluids of a wide range of plant pathogenic fungi e.g. *Botrytis* spp., *Fusarium* spp., *Helminthosporium* spp. and *Pythium* spp. suggesting that many fungi excrete cutin hydrolyzing enzyme(s).

The observations of water-soaked lesions on the carrot roots 2 days after inoculation with *P. violae* might suggest that the production of cell wall degrading enzymes, in particular "toxic" endo-pectinases, play an important role in pathogenesis.

The cell wall of carrot is responsible for the firmness and cohesiveness of the root tissues. Massiot et al. (1988) working on the isolation and characterisation of the purified cell walls of carrot showed that in mature carrot, cell walls contain all the polysaccharides usually found in dicotyledonous cell walls. These were cellulose (*ca.* 25%), hemicelluloses (10-15%) as xyloglucans, xylans and mannans and pectic material with associated arabinose and galactose containing polysaccharides (45-50%) and complexed with proteins and polyphenolic material. Pectins were highly methyl-esterified and rich in galacturonic acids.

In addition to cell wall degrading enzymes, *Pythium* spp. may produce toxins that cause cellular disruption in advance of tissue invasion. Heat labile toxic metabolites and hydrolytic enzymes were found by Sadik et al. (1983) in culture filtrates taken from virulent isolates of *P. aphanidermatum*. When culture filtrates were introduced into the tomato shoots, they induced wilt and caused softening of the tomato shoots. Culture filtrates of *P. butleri* have also been reported to induce severe toxic effects on the seedlings of belladonna 6 hrs after seedlings were placed in culture filtrate (Janardhanan and Husain, 1974).

Pathogen-produced plant hormones which may be involved in symptom expression have been shown from *Pythium* spp. cultures (Martin, 1995). Blok (1970) demonstrated that roots of flax infected at the tip by *P. sylvaticum* had decreased growth but swollen tips and symptoms which resembled those caused by indole acetic acid (IAA).

Chérif et al. (1991) studied ultrastructural and cytochemical events of fungal development and host reactions in cucumber plants infected by a virulent strain of *P. ultimum.* He showed that infection occurred in 2-7 hrs, the fungus was limited to the intercellular and intracellular regions of the cortical area in roots by 48 hrs. Protoplasm collapsed and different organelles disintegrated in plant tissues following the colonisation of vascular stele, endodermis, the pericycle and the parenchyma cells.

The penetration stage of *P. violae* in the light and electron microscopy was reported by Byrd (1988) who found that *P. violae* penetrated periderm directly, regardless of the soil treatment and the age of the carrot. The findings which showed superficial invasion of *P. violae*, collapse of periderm cells and lignification of cell walls, confirmed the earlier microscopy of Guba et al. (1961) and Perry and Harrison (1979a).

The role of cell wall degrading enzymes in cavity spot formation of carrot, as mentioned above, has been examined in this study.

3.3. POWDERY MILDEW DISEASE CAUSED BY *Erysiphe heraclei*

Erysiphaceae, the powdery mildews or white mildews, are obligate biotrophic parasites of Angiosperms and a very important group of plant pathogens. The term powdery mildew is derived from their white mealy appearance on infected plant parts (Webster, 1991).

Powdery mildew *(Erysiphe heraclei)* infects umbelliferous crops including celery, carrot, coriander, fennel and parsnip and occurs in regions with warm and dry climates such as Mediterranean countries, India and North Carolina (Dixon, 1981; Geary and Wall, 1976; Jenkins et al., 1985; Smith and Clark, 1988). Powdery mildew which appears as large areas of diffuse white mycelium on both leaf lamina and petioles is one of the most important leaf diseases of carrot in the field and causes serious damage under favourable environmental conditions.

The branched mycelium remains superficial on the surface of the host epidermis. Asexual reproduction occurs with cylindrical conidia, about 35-45×15-20 μm. Conidia are produced singly, rarely in short chains. Sexual reproduction occurs with cleistothecia which are initially globular in shape but then become flattened, about 85-120 μm in diameter. Between three and ten elliptical or globular asci within the cleistothecium are formed, each containing between three and six ascospores. Conidia germinate with unforked germ tubes. *E. heraclei* can be distinguished from other *Erysiphe* species by its abundant and branched cleistothecial appendages and its elongated cylindrical conidia (Dixon, 1981; Jenkins et al., 1985).

On carrot, *E. heraclei* appears to attack mature leaves first, which may become chlorotic and senescent, then spreads to younger leaves. Young plants that become infected have reduced vigour and may even be killed (Dixon, 1981; Geary and Wall, 1976).

There is virtually no documented evidence on the existence of cultivars resistant to *Erysiphe heraclei.* Lebeda and Coufal (1987) tested 111 carrot varieties under field conditions with natural mildew inoculation. Only one variety, "Gavrilovskaja" from former USSR, was absolutely free from mildew under these conditions. About 50% of their collection was moderately vulnerable.

Although control of the disease has been achieved with systemic chemicals such as benomyl, carbendazim and thiophanate-methyl or protectants such as maneb plus morestan, triphenyltin acetate or triphenyltin hydroxide (Dixon, 1981), resistant cultivars should be used in order to reduce the cost of chemicals. Therefore, the possibility of selecting resistant cultivars by using tissue culture techniques was investigated in this study.

4. SOMACLONAL VARIATION AND DISEASE RESISTANCE

Any type of variation induced by tissue culture techniques and the plants regenerated from any form of cell culture displaying genetic variation were described as somaclonal variation by Larkin and Scowcroft (1981). The frequency of somaclonal variation has been estimated as 30-40% for the number of regenerated plants showing some type of variation and from 0.2 to 3% for variation in a desirable trait (Daub, 1986; Larkin and Scowcroft, 1981; Lorz and Scowcroft, 1983). As a result of somaclonal variation many agronomically useful variants e.g. disease resistance have been obtained.

In the late 1960s, the first evidence for the variation which spontaneously originated amongst cultured sugarcane cells was noticed by Heinz et al. (1969). Although the study was preliminary and only the initial regenerants were tested, the resistance was shown to be passed on to the progeny.

Many types of genetic changes occur in somaclonal variation including alterations in DNA sequence e.g. single gene mutation, transposition, amplification; in gross chromosome structure e.g. duplications, translocations, deletions; in chromosome number e.g. polyploidy or aneuploidy; and in chloroplast or mitochondrial genomes (Bayliss, 1980; Karp, 1990; McCoy et al., 1982). These types of changes are stable through succeeding generations. However, the variation exposed as a result of a tissue culture cycle can be non-heritable (epigenetic) which would not be transmitted through meiosis and it may be reversible during the life of a plant. Hence it is worthless for sexually propagated plant production. Changes have also been identified that are both heritable and unstable (Karp, 1990).

Somaclonal variation can be influenced by a combination of factors. These include the species and genotype, the ploidy level, tissue culture procedures employed, time and frequency of subculture, the source of explant and the composition of the culture medium (Ahmed and Sági, 1993; Daub, 1986; Karp, 1995; Peschke and Phillips, 1992).

Explant tissue may not be genetically homogeneous and heterogenecity may be magnified by the proliferation of differing cell types. Thus mesophyll protoplasts of Su/su (heterozygous, light green) tobacco plants cultured by Lorz and Scowcroft (1983) gave 2,156 calli, of which 79 produced plants. Of these 79 colonies, 25% were phenotypically homogeneous (Su/Su dark green, su/su pale) and the remaining 75% of colonies were heterogeneous. These findings point to either extremely early mutational

events or to variation preexisting in the protoplast. van den Bulk et al. (1990) working on the effect of explant type on the variation of tomato cultures found little difference in regenerated plants derived from different explants except from hypocotyls which produced 58% polyploid cells.

Mutagenic action of media components, especially hormones, has often been demonstrated. Bayliss (1973; 1977a) illustrated that in diploid suspension cultures of carrot which were grown for 90 weeks, 0.1 mg/l 2,4-D caused significantly higher frequency of multipolar anaphases and lagging chromosomes by spindle failure. Later work showed that above 30 mg/l 2,4-D completely prevented spindle formation. The frequent establishment of fresh cultures, the use of suitable medium and subculture regimes can maintain clonal fidelity in both cultures and regenerated plants (Krikorian, 1982). In sugarcane, regenerated somaclones resistant to sugarcane mosaic virus were obtained from a susceptible variety by increasing the number of subcultures of the embryogenic callus in MS medium supplemented with 3 mg/l of 2,4-D. DNA fingerprint results showed that resistant somaclones had different genetic constitutions from the maternal line (Oropeza et al., 1995).

Somaclonal variation can be influenced by the genotype of the donor plants. Plants regenerated from two cultivars of oat, Lodi and Tippecanoe produced different frequencies of cytogenetically abnormal plants. 49% of Lodi regenerated plants and 12% of Tippecanoe regenerated plants were abnormal after 4 months in culture (McCoy et al., 1982). The frequency of somaclonal variation in poplars of the *Leuce* section (8%) was higher than in those of the *Aigeiros* and *Tacamahaco* sections (1%). It was shown in this study that regenerated variants were tetraploid or heteroploid while original clones were all diploid (Antonetti and Pinon, 1993). The genetic structure of source plants that already show low or moderate levels of resistance can affect successful

selection for disease resistance (Daub, 1986). In celery, a much higher frequency of plants highly resistant to Fusarium yellows (*Fusarium oxysporum* f. sp. *apii*) was regenerated from embryogenic suspension cells of a moderately resistant cultivar than from highly susceptible source material (Wright and Lacy, 1988). Recently, Sebastiani and co-workers (1994) have also found similar results in potato by using callus cultures induced from stem explants of a cultivar (Désirée) tolerant to *Verticillium dahliae*. *Verticillium* culture filtrates were applied to single node cuttings for *in vitro* selection of resistant clones and then regenerants were infected with fungal conidia to confirm the resistance.

There is evidence that the length of the culture period has a significant effect on the extent of variation generated during culture. Prolonged suspension cultures of carrot generated higher frequencies of tetraploidy, octoploidy and aneuploidy within the cells, but it was also associated with reduced embryogenic potential (Smith and Street, 1974). Long term maintenance of carrot callus cultures on medium containing 2,4-D also resulted in entirely aneuploid cells in callus. However, these callus cultures lost their ability to form embryos (Halperin, 1966). In oats (*Avena sativa* L.), McCoy et al. (1982) noticed that the frequency of cytogenetically abnormal, regenerated plants increased dramatically with increased time in culture. Frequency of observable chromosome aberrations (trisomics, monosomics, interchanges and plants with deficient chromosomes) increased in one cultivar from 49% after 4 months of culture to 88% after 20 months. Some strains of *Pisum sativum*, after prolonged period of subculture, showed a wide range of chromosome numbers at higher ploidy levels but completely lacked diploidy. The loss in root regeneration capacity was related to the increase in abnormality of chromosomal constitution (Torrey, 1967). Higher level of resistance to sugarcane eyespot (*Helminthosporium sacchari*) toxin in regenerated plantlets was

obtained with prolonged callus cultures (Larkin and Scowcroft, 1983). Benzion and Phillips (1988) showed that the frequency of cytogenetically abnormal regenerated maize plants, increased with culture age. They concluded that the age effect was not due to an increased mutation rate, but was due to mutational events that occurred throughout culture development with subsequent maintenance and accumulation of aberrant cells over time. Morphogenetic callus was diploid while non-morphogenic callus was found to contain high frequencies of aneuploidy, triploidy, tetraploidy and octoploidy in barley. As a result of increased chromosome numbers, regeneration acted as a barrier against the more extreme variants as a loss of organogenesis is related to a high degree of aneuploidy (Singh, 1986). However, it was also demonstrated in potato that calluses exhibiting high levels of aneuploidy are still capable of shoot regeneration, giving wide ranges of chromosome numbers in regenerated plants (Karp et al., 1984). Although embryos and plants were produced from long-term carrot cultures, these plants were either sterile or formed very few seeds which did not survive after germination (Sussex and Frei, 1968). Recently, somatic segregation as a part of genetic variation was shown in carrot hypocotyl explant by Giorgetti et al. (1995). The meiosis-like divisions at 1-3% was observed in hypocotyl explants, in the presence of auxin. Cytological investigations of carrot cell lines which were kept long term in culture revealed the ranges of chromosome numbers e.g. new levels of ploidy and novel chromosome numbers. Mainly aberrant divisions resulted in two haploid prophases and metaphases, appeared as a segregational process, during which the chromosome number is halved from 2n (diploid embryogenic cell line) to n (haploid cell line) (Ronchi et al., 1992).

The tissue culture procedure employed can also affect variation. Meristems cultured without a state of dedifferentiation produced little or no variation in contrast to when a dedifferentiated state was induced (Bayliss, 1980; Karp and Bright, 1985).

Protoplast regenerants tend to be more variable than those produced directly from leaf or stem tissues. Dudits et al. (1976) demonstrated that carrot protoplasts (isolated from cell cultures) treated with polyethylene glycol (PEG) to induce protoplast fusion resulted in a higher frequency of tetraploid and hexaploid chromosomal structures in regenerated plants (41.2%) than those grown from untreated protoplasts (16%) and from the original cells (6.6%).

Cultured carrot cells exhibited substantial variation in chromosome number, both ploidy and aneuploidy and chromosome morphology, whereas regenerated plants were diploid, with the exception of a few tetraploids and they showed no cytological abnormalities (Mitra et al., 1960).

The length of interval between subcultures may also be important in somaclonal variation. Evans and Gamborg (1982) illustrated that short subculture intervals were necessary for maintenance of chromosome stability in cell suspensions of *Nicotiana* spp. Suspension cultures subcultured to fresh medium at 7-day intervals showed a notable decline in the frequency of tetraploid cells within the diploid culture of carrot. Linear growth and stationary phase periods of carrot suspension cultures were eliminated by 7-day subculture regime while maximum growth rate and mitotic index of cultures did not change (Bayliss, 1975; 1977b).

Phenotypic variants have also been observed among carrot plants regenerated from callus culture, with erect stems and green, thick and short leaves and broad petioles. However, these changes were epigenetic rather than genetic (Ibrahim, 1969).

4.1. *In Vitro* SELECTION FOR DISEASE RESISTANCE

Control of disease with chemicals can be expensive, can result in problems of residues, can cause environmental pollution and pathogens may develop resistance; also it may not be suitable to low-income farming systems. Control is best achieved with resistant cultivars but there are none available against the three diseases of carrot considered in this study. However, inefficient screening procedures and possibly lack of genetic variation can restrict progress in obtaining disease resistance *via* conventional breeding systems. Tissue culture methods can be used to reveal sometimes new genetic variations in intact plants and offer great opportunity for selection of resistant plants at the cellular level. In particular, somaclonal variation offers a great opportunity to increase the genetic variation of crops and can be applied for selection of disease resistance to pathogens (Jones, 1990). The advantages of tissue culture systems to select for disease resistant varieties are manifold: i) control of nutritional and environmental factors which may allow for selection of small increments in disease resistance; ii) homogeneous plant cells can be subjected to selective agents; iii) control of pathogen inoculum levels; iv) ability to modify the nature of the host-pathogen interaction by altering components of the growth medium; v) the reduction of time for selection and space for maintenance of selected cells (Ahmed and Sági, 1993; Miller and Maxwell, 1983). However, a disadvantage introduced by tissue culture is that plant cells grown in culture conditions may not be genetically and physiologically the same as cells of intact plants (Ingram, 1977).

Disease resistance is regulated by monogenic (one or several major genes are involved and resistance alleles are usually dominant) and polygenic (in which a large number of genes with small individual additive effects) systems.

The development of an *in vitro* selection programme can be split into three principal stages. These are: i) the production of the genetic variation; ii) the operation of selection (involving choice of the appropriate selective unit, e.g. callus or protoplast, selective agent e.g. toxin of pathogen, selection procedure, culture conditions); iii) analysis of the selected regenerants (level of disease resistance, stability, agronomic characteristics).

Somaclonal variation and successful selection of somaclonal variants with an increased disease resistance or tolerance at the plant level has now been recorded for many crop species (Larkin and Scowcroft, 1981; Sacristan, 1986; van den Bulk, 1991).

Callus tissue culture systems have widely been used for studies of race specific, race non-specific and non-host resistance (Miller and Maxwell, 1983) while suspension cultures are commonly used in studying resistance-related genes including phytoalexin induction and biosynthesis (Buiatti and Ingram, 1991).

4.1.1. Selection Using Obligate Parasite

Tissue culture techniques offers a great opportunity for studying obligate biotrophic pathogens such as powdery mildews (*Erysiphales*), downy mildews (*Peronosporaceae, Sclerosporaceae*), white rusts (*Albuginaceae*), rusts (*Uredinales*) and *Plasmodiophorales* which can not be grown on artificial medium (Ingram, 1977; Miller, 1985). The use of dual cultures of a biotrophic fungus and callus tissue derived from a host has the advantage for maintenance of contaminant free inoculum, cloning of pathogen isolates, safe international transport of isolates, establishment of axenic cultures, earlier testing of plants for resistance to pathogens and also provides for studies of host parasite interactions without contamination by other microorganisms (Ingram, 1977).

However, Ingram (1977) showed that co-cultures of callus tissue from resistant and susceptible host genotypes with obligate fungal parasites did not differentially express resistance *in vitro*.

Successful establishment of dual cultures is dependent on the balance between the growth rates of the plant tissues and pathogen. Both callus tissue and hyphae should continue to grow without one of them becoming dominant during subculture. Dual cultures of sugar beet and downy mildew pathogen *Peronospora farinosa* f. sp. *betae* were established successfully by placing a diseased explant onto one of the callus tissue culture media or at sporulation stage, placing an uncontaminated explant onto the surface of the callus tissues (Ingram, 1980; Miller, 1985). *In vitro* sugarcane tissue cultures facilitated earlier testing of plants for resistance to the smut fungus (*Ustilago scitaminea)* (Fereol, 1984). In many studies, particularly of rusts, resistance *in vivo* against such pathogens was not expressed in callus cultures. Jacobi (1982) reported that loblolly pine callus cultures suppressed mycelial growth of *Cronartium fusiforme* derived from both basidiospores and urediospores while abundant growth was seen on noncallused loblolly pine seedlings. Many different methods (e.g. aerial mycelium, urediospores of rust fungus, predifferentiated urediospores with germ tubes) tested to inoculate leaf callus tissues of snapdragon and sunflower were unsuccessful (Maheshwari et al., 1967). The general failure of the dual cultures of obligate parasites and callus tissues for selection of disease resistant plants could be explained by the absence from calli of cuticle and epidermis which might be involved in host recognition or secretion of extracellular substances which inhibit germination of fungus from callus tissues (Ingram, 1977; Maheshwari et al., 1967).

However, Msikita and Wilkinson (1994) reporting on the selection of powdery mildew resistant variants in Kentucky bluegrass genotypes, obtained markedly increased resistance to *Erysiphe graminis* in regenerated plants derived from embryogenic callus.

4.1.2. Selection Using Facultative Parasites

Most of the *in vitro* studies on the exploration of disease resistance have involved facultative fungal and bacterial pathogens and their interactions with callus tissue, suspension cultures, isolated cells, protoplasts or plantlets.

The successful application of *in vitro* selection for disease resistance was first shown by Carlson (1973) for wildfire disease of tobacco, caused by *Pseudomonas syringae pv. tabaci.* Haploid plant cells and protoplasts treated with EMS (ethylmethanesulfonate) were exposed to methionine sulphoximine which elicits similar chlorotic haloes on tobacco leaves to the toxin that is a structural analogue of methionine produced by *P. syringae* pv. *tabaci.* Regenerated somaclones from three surviving calluses showed no or less chlorosis than the original plants.

Sugarcane was the first plant in which the possible usefulness of the variability originating in cell culture for recovering plants with increased disease resistance was recognized. Somaclones regenerated from either callus or suspension cultures of the susceptible cultivars were shown to be more resistant than the original material against eyespot (*Helminthosporium sacchari*), downy mildew (*Sclerospora sacchari*) and Fiji disease (Heinz et al., 1977; Krishnamurthi and Tlaskal, 1974; Sacristan, 1986). Although the heritability of these diseases was not known, vegetative propagation was used to maintain resistant subclones. Sugarcane cultivar "Ono" resistant to Fiji disease was released following the research of Krishnamurthi and Tlaskal (1974). Cultivar "Ono" was derived from somaclonal variation and identified by screening at the plant

level. A cultivar of sweet potato isolated from meristem tip culture derived clones has also been released. This cultivar "Scarlet" yielded similar to the those of the parent cultivar and also disease resistance characteristics were similar to parent cultivar, but has darker and more stable skin colour, texture and flavour, which is a much more desirable for baking (Moyer and Collins, 1983). The recovery of disease resistant plants from *in vitro* cultures of the vegetatively propagated crop, potato, has also been reported. Clones derived from protoplast populations showed resistance to *Phytophthora infestans* and *Alternaria solani* (Shepard et al., 1980).

Varying results have been obtained when pathogens were used as the selection agent. Sacristan (1982) was unable to identify increases in resistance to *Phoma lingam* in somaclones derived from callus cultures of haploid rape (*Brassica napus*) by inoculating cultures with spores of the pathogen. Investigations demonstrated that similar responses were shown to pathogen attack in intact plants and in callus culture systems. Haberlach et al. (1978) reported that *Phytophthora parasitica* var. *nicotianae* (blank shank) tested in callus derived from resistant plants showed little or no fungal colonization compared to susceptible tissues. Expression at the cell culture level of the monogenic resistance of tobacco to blank shank was noted only under certain phytohormone levels in the culture medium. Host-parasite interaction in alfalfa to *Phytophthora megasperma* was examined with calluses derived from resistant and susceptible hosts and fungus which was nonpathogenic to the host. These studies revealed that susceptible callus tissue was infected heavily while aerial hypha was limited to the top of the callus on the nonhost resistant and resistant cultivar interactions (Miller et al., 1984). The high frequency of celery plants resistant to *Fusarium* using tissue cultures techniques by plating small regenerated shoots on a medium precolonized with *Fusarium oxysporum* f. sp. *apii*, race 2 were obtained by Pullman and Rappaport

(1983). In later work it was apparent that increased resistance to *F. oxysporum* f. sp. *apii*, race 2 among the somaclonal variants of celery, UCT3 was transmitted to progenies (Heath-Pagliuso and Rappaport, 1990). Recently, Dan and Stephens (1995) working on the development of asparagus somaclones with high levels of resistance to *F. oxysporum* reported that regenerated plants were significantly more resistant than the parental cultivar.

Cultured cells of tobacco showed an hypersensitive-like response (HR) to incompatible pathogens in suspension cultures. Inoculation of suspension cultured tobacco cells with the incompatible *Pseudomonas syringae* pv. *pisi* illustrated that suspension cultured tobacco cells could provide a model system for studies of hypersensitivity (Atkinson et al., 1985). In an other work, Matthyse (1987) inoculated suspension cultures of carrot and tobacco with the incompatible bacterium *P. syringae* pv. *phaseolicola* and also observed a hypersensitive reaction.

4.1.3. Selection *in vitro* with Toxins and Culture Filtrates

Plants with novel resistance might be regenerated after the selection of toxin resistant cells from cultivars which are sensitive to the toxin. Many facultative fungal and bacterial pathogens produce a great variety of low molecular weight toxic metabolites *in vitro* and *in vivo*. These are classified into host selective and non-host selective toxins (Buiatti and Ingram, 1991). Toxic metabolites produced by pathogens have been used frequently as selective agents *in vitro* (Binarová et al., 1990; Hammerschlag, 1988; Hammerschlag et al., 1994; Sacristan, 1982). Positive correlations have been shown between resistance to pathogens at the plant level and insensitivity to the toxin at the cellular level with several host specific toxins (Carlson, 1973; Larkin and Scowcroft, 1983). However, it has been shown that resistance to host

specific toxins in some species was not expressed at the cellular level. Wolf and Earle (1990) attempted selection following toxin treatment, but were unsuccessful in recovering callus of maize resistant to the *Helminthosporium carbonum* race 1 toxin. The effect of AAL toxins produced by *Alternaria alternata* f. sp. *lycopersici* were examined on leaves, leaf disks, roots, calli, suspension cells and protoplasts of resistant and susceptible tomato genotypes. The typical symptoms were observed on leaf and root while toxins did not affect calli, suspension cells, protoplasts or shoots induced from leaf disks. These results suggest that differential toxin effects were related to phytohormone levels of plant parts (Witsenboer et al., 1988). Similar results were also reported by Newsholme et al. (1989). The secondary embryoids of *Brassica napus* ssp. *oleifera* were treated with unpurified culture filtrates and a putative pathotoxin, Sirodesmin PL of *Leptosphaeria maculans* to select toxin resistant plants. Regenerated plants were found to be similar to plants from unselected or control cultures in their resistance to *L. maculans* and in some cases, regenerated plants appeared to be more susceptible to the pathogen than plants grown from seeds.

Disease resistant plants may be obtained after the selection of toxin resistant cells from genotypes which are sensitive to the toxin. Non-host specific toxins are usually regarded as virulence factors, i.e. compounds which increase the extent of the disease symptoms. However, plants resistant to these toxins may probably not show complete resistance to the pathogen. Selection for bacterial blight with the non-host specific toxin, syringomycin produced by *P. syringae* pv. *syringae*, in wheat callus cultures derived from immature embryos resulted in regenerated plants with an increased resistance (Pauly et al., 1987).

Relatively few toxins have been conclusively shown to play a significant role in plant disease. Understanding the mode of action and properties of the toxin are

important factors for selection of toxin resistant cells. The importance of the mode of action and properties of toxins were demonstrated by Daub (1982) who failed to select tobacco and sugar beet cells resistant to the non-specific toxin cercosporin. The occurrence of peroxidation of polyunsaturated fatty acids was observed when plant cells were treated with cercosporin i.e. the toxin altered the membrane structure and fluidity of cells and resulted in rapid increased in membrane leakiness.

A toxic metabolite in culture filtrate produced by *Xanthomonas campestris* pv. *pruni*; was claimed to be involved in bacterial spot development of peach. Callus cultures initiated from the bacterial spot susceptible cv. Sunhigh were inoculated with the toxic culture filtrate for several selection cycles. Peach plants were regenerated from the surviving calli and two out of four selected clones were significantly more resistant to bacterial spot than the original cv. Sunhigh in the green house and also under tested field conditions (Hammerschlag et al., 1994).

In many studies, unpurified or partially purified culture filtrates have been the most widely used *in vitro* selective agents (MacDonald and Ingram, 1986; Sacristan, 1982). One of the dangers of this approach is that toxicity to which resistance is being selected may be due to components of the pathogen culture medium and not due to a pathogen produced toxin, or to "toxins" not involved in pathogenicity. This strategy was first described by Behnke (1979). He studied crude culture filtrates of two potato (*Solanum tuberosum*) pathogens, *Phytophthora infestans* and *Fusarium oxysporum*, to select resistant callus. In the case of *P. infestans*, selected callus was resistant to all four pathotypes of the fungus although the crude filtrate came from only one pathotype. The resistance was stable in the absence of the filtrate and also retained in callus derived from some regenerated plants. Callus and embryogenic cultures of *B. napus* have been

selected for growth in the presence of a crude culture filtrate of *Phoma lingam*. Some regenerated plants exhibited increased resistance to the pathogen.

A highly embryogenic cell suspension culture of alfalfa obtained from a genotype susceptible to *Fusarium oxysporum* was used in the study of selection for resistance to culture filtrates of *Fusarium* spp. 12-20% more regenerated plants exhibited increased resistance to fungus than plants regenerated from a control cell line (Binarová et al., 1990). Using callus cultures of susceptible alfalfa cell lines, heritable resistance to toxic components produced by *Fusarium oxysporum* was selected at the plant level (Hartman et al., 1984).

Antonetti and Pinon (1993) recently demonstrated that callus from poplars that had survived exposure to increasing concentrations of toxins from *Hypoxylon mammatum* gave rise to a toxin-tolerant line from which toxin tolerant plants were regenerated.

The occurrence of somaclonal variation and selection of disease resistant plants including those from embryogenic suspension cultures have been reported in various studies as explained earlier. However, there is no information about resistance to *Erwinia carotovora, Pythium violae* and *Erysiphe heraclei* within known carrot varieties. Therefore, it was attempted to use highly embryogenic suspension cultures of carrot for selection for resistance to either soft rot bacteria, *Erwinia carotovora* or cavity spot, *Pythium violae* or to their products in this study. Callus cultures were applied for screening of *Erysiphe heraclei* resistant carrot plants since *E. heraclei* is an obligate parasite and therefore can not be grown in culture.

5. AIMS

The main objectives of this study were:

1. To establish a suitable carrot tissue culture system to use for selection of disease resistance to three major pathogens of carrot.
2. To develop suitable inoculation techniques with the three pathogens of carrot.
3. To test disease resistance and/or tolerance to pathogens of regenerated plants.
4. To evaluate the use of embryogenic suspension cells as a means of regenerating putative, novel disease resistant plants following challenge with *Ecc* wild type and mutants and with *P. violae.*
5. To examine the heritability of the regenerated plants if the resistance to any of the pathogens was obtained.

CHAPTER 2

MATERIALS AND METHODS

1. CARROT TISSUE CULTURE

1.1. PLANT MATERIAL

Four domestic F_1 hybrid carrot cultivars, Morot Duke, Morot Favor, Morot Ingot and Morot Rondino were received as seeds from Hammenhögs (Svalof-AB. Hammenhögs-Sweden). Two open-pollinated commercial cultivars, New Red Intermediate (NRI-92) and Autumn King, were obtained as seeds from Suttons and seeds of five cultivars, Gavrilovskaja (resistant), Guerenda (susceptible), Bertina (susceptible), Erstling (resistant) and Danro (susceptible) with claimed different levels of resistance to powdery mildew were supplied by Dr. Ales Lebeda, Zuchtstation Smrzice laboratory, Czeck Republic (pers. comm.). Taproots of carrot cv. Burton were collected from Mortimer's Farm, Bromham, Wilts. Carrot cv. Morot Duke taproots were also produced at Bathampton Field Station (University of Bath) and carrot roots

(cultivar unknown) for inoculation with *Pythium violae* were obtained from the local market.

1.2. PREPARATION AND SURFACE STERILIZATION OF EXPLANTS

Carrot seedlings were used to initiate all cultures. Before surface sterilization the seeds were rinsed 10 times in sterile distilled water containing one drop of wetting agent (Tween-80). The surfaces of the seeds were sterilized by agitation with 15% NaOCl containing one drop of Tween-80 for 30 min with a magnetic stirrer, after which the NaOCl was removed by rinsing five times with sterile distilled water. Seeds were then placed with forceps onto petri dishes (20 seeds/dish) which contained half strength MS basal medium (Imperial Ltd.) supplemented with 2% (w/v) sucrose. After germination, hypocotyls and cotyledons were used as explants; a sterile needle and forceps were used for excision. All procedures were performed under sterile conditions provided by a laminar flow cabinet (Microflow Path-finder Ltd.).

1.3. CULTURE MEDIA

The basal medium of Murashige and Skoog (1962) was used for callus and suspension cultures. Sucrose as the carbon source was routinely used in all media at 2% (w/v). Heat stable plant growth regulators such as 2,4-dichlorophenoxyacetic acid (2,4-D), were added before the medium was autoclaved, whereas the heat labile growth regulators and antibiotics were filter-sterilized through a membrane filter of pore size 0.2 μm (Sartorius Ltd.) and added to the medium as it cooled (50 °C). The pH was adjusted to 5.7 with 1 M NaOH or 1 M HCl. 0.7% (w/v) Oxoid No. 3 agar was added for the production of semi-solid media. The media were dispensed to screw-top bottles (100 ml, 250 ml or 1000 ml as appropriate) and autoclaved at 1.5 bar/120 °C for 15 min. After cooling, semi-solid media were dispensed into 9 cm diameter plastic sterile petri

dishes (Sterilin Ltd.) at 25 ml per dish and liquid media at 25 ml per 100 ml conical flask and 50 ml per 250 ml conical flask.

1.4. ESTABLISHMENT OF CALLUS AND SUSPENSION CULTURES

1.4.1. Initiation, Subculture and Maintenance of Callus Cultures

The callus cultures were initiated and maintained in the following way: hypocotyl explants, 4-6 mm in length and cotyledon explants from the same seedlings were transferred onto petri dishes containing Murashige and Skoog (MS) medium with 2% (w/v) sucrose and 0.7% (w/v) Oxoid No. 3 agar supplemented with 2,4-D (concentrations indicated in Results sections) for the initiation and maintenance of callus cultures. Petri dishes were sealed with Parafilm M. (American Can. Co.) in order to prevent desiccation.

All cultures were incubated at 25 ± 1 °C either under light with a 16 hrs photoperiod (30 μmol m^{-2} s^{-1}) or in dark conditions.

Stock callus cultures were subcultured regularly at monthly intervals onto the fresh MS medium containing 2% (w/v) sucrose and 0.5 μM 2,4-D.

1.4.2. Initiation, Subculture and Maintenance of Suspension Cultures

Four cultivars were cultured for suspension cultures: Morot Duke, Morot Favor, Morot Ingot and Morot Rondino.

Callus was induced from hypocotyl explants and maintained on MS medium supplemented with 2% (w/v) sucrose and either 0.5μM 2,4-D or 5μM 2,4-D and adjusted to pH 5.7-5.8 and solidified by the addition of 0.7% (w/v) agar.

The cultures were initiated from eight-week-old callus by cutting 0.1-0.2 g pieces and transferring into 25 ml MS liquid medium containing 2% (w/v) sucrose and 0.5μM 2,4-D in 100 ml conical flasks. After 14 days, 5 ml aliquots of the suspension

cultures were removed by a sterile pipette with a 0.2 cm diameter orifice to inoculate 45 ml of fresh medium in 250 ml conical flasks. Such suspensions were subcultured in the same manner every 14 days.

Conical flasks were covered with two layers of aluminum foil and agitated on an orbital shaker in an incubator (Gallenkamp and Co., Ltd.) at 100 rpm. These flasks were incubated at 25 ± 1 °C with 16 hrs daylight (30 μmol m^{-2} s^{-1}) per day from cool white fluorescent tubes.

1.4.3. Subculture Regime for Stock Cell Suspension Cultures

A standard regime based on the primary experiment described in Chapter 3.I, Section 3.1 was established for suspension cultures of Morot Duke. The growth of the suspension cultures was measured by determining total cell volume after sedimentation (CVS) at two day intervals in an apparatus designed to tilt a 250 ml conical flask to an angle of 60° from the horizontal. The flasks were placed in the apparatus and left for 5 minutes for sedimentation of the cells and a ruler fixed in the apparatus was used to measure the height of cells along the lower wall of the flasks (Plate 1) (Blom et al., 1992). The relationship between the heights and the equivalent volumes of cells was established by reference to a calibration curve prepared with known volumes of water. To find the relationship between height and CVS, a regression analysis based on the volumes of the water samples was prepared (Appendix 1).

Carrot culture system which has been developed for use of subsequent co-culture studies is shown schematically in Figure 2.1 and also used for regeneration of somaclones (i.e. whole plants from callus and suspension cultures).

Plate 1: **Measurement of cell volume after sedimentation (CVS), 5 min after conical flask is placed in apparatus. The height of the cells along the glass wall of the flask was measured by the ruler fixed in the apparatus**

Plate 2: **Embryogenic suspension cultures of carrot cv. Morot Duke after fractionation by sieving through 100 μm nylon sieve. Note the presence of dense embryogenic units and non-cytoplasmic, translucent non-embryogenic units. The white arrows show non-embryogenic cells, black arrows show embryogenic cells (×175)**

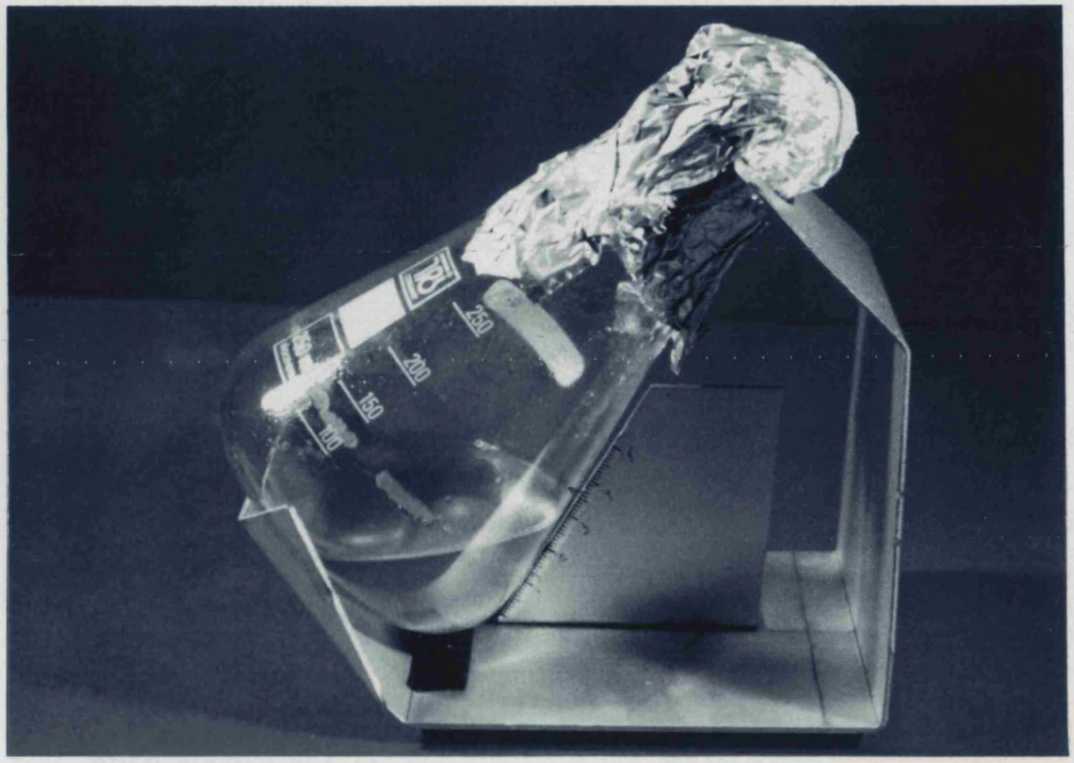


Plate 1

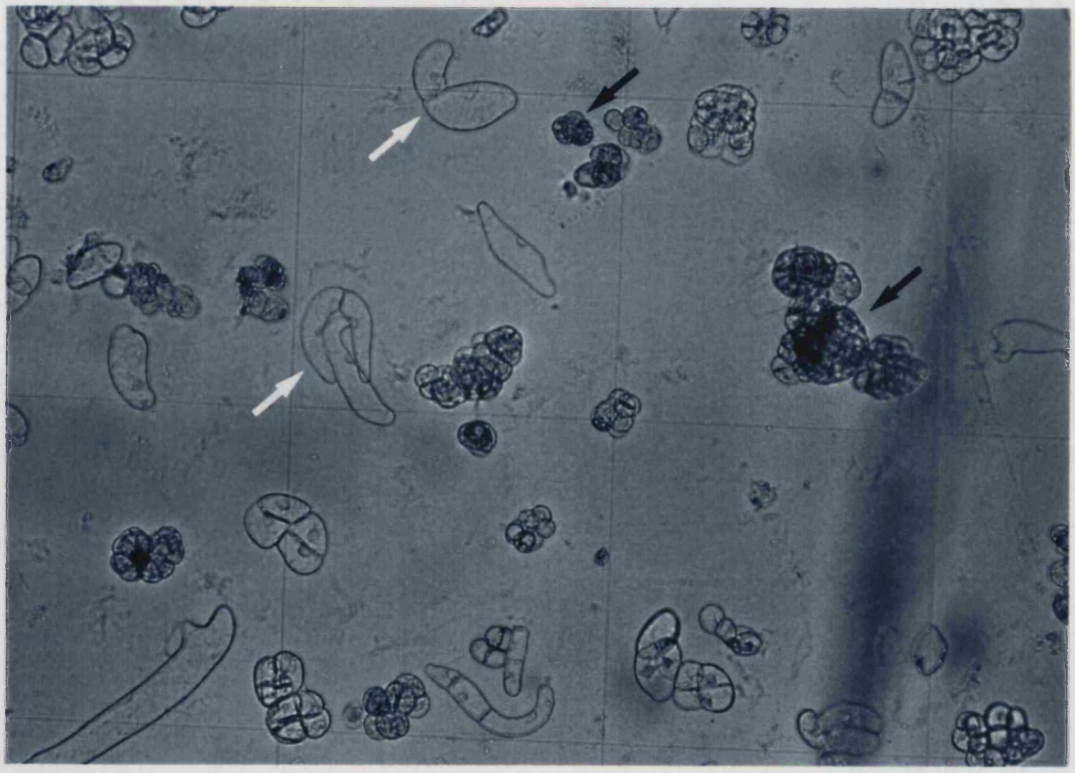


Plate 2

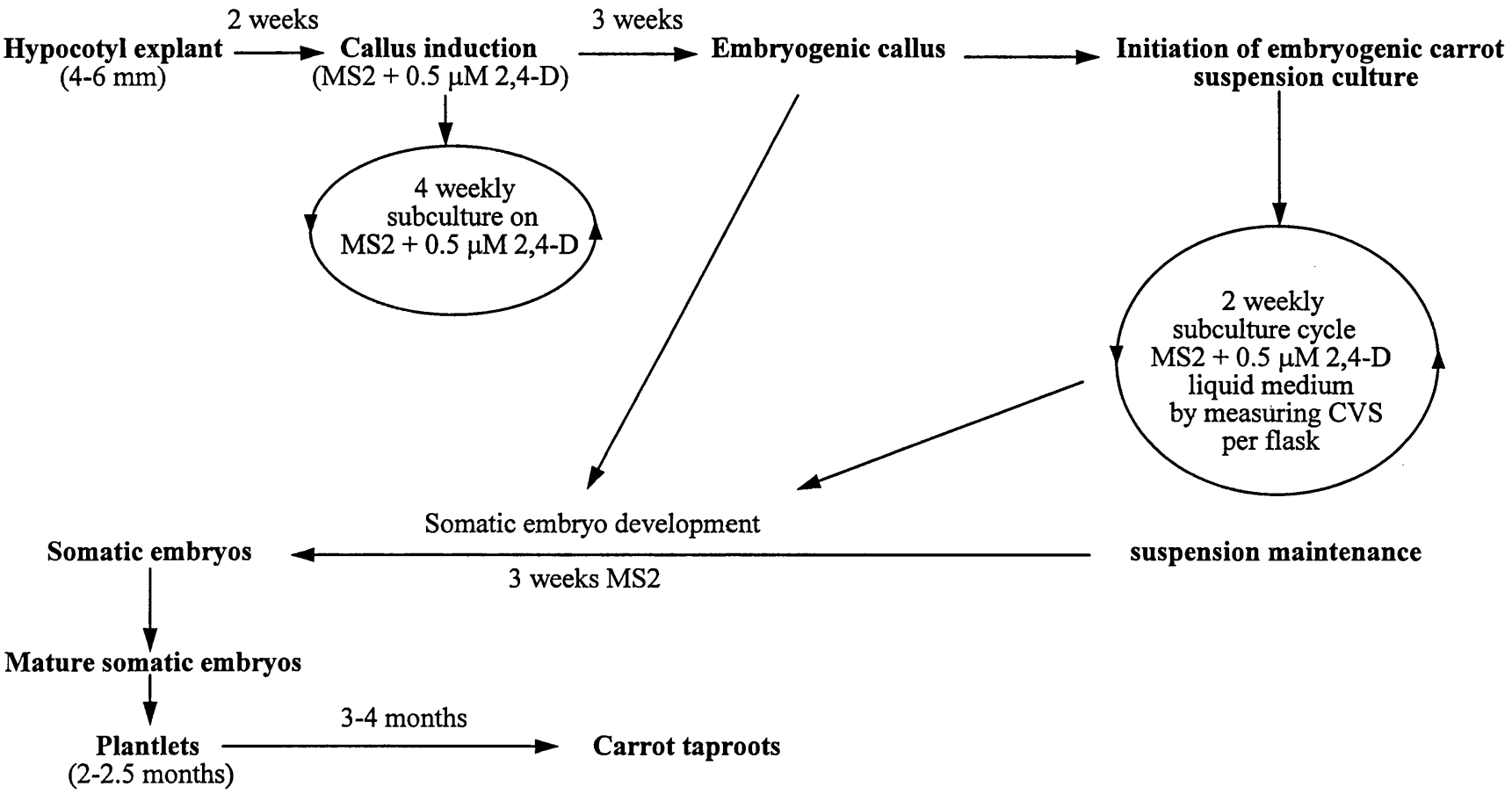


Figure 2.1: Schematic representation of initiation, maintenance and regeneration of embryogenic cultures of carrot.

1.4.4. Preparation of Carrot Cell Suspension Culture for Co-Culture

The population of the cells in a suspension culture is usually heterogeneous in terms of sizes of the cell units, in contrast to the cell populations used in co-culture experiments which should be as homogeneous as possible. The cultures were therefore passed through a series of nylon sieves of different mesh sizes to obtain fractions with different cell unit sizes (Fujimura and Komamine, 1979a).

Ten-day-old cells of cv. Morot Duke (Plate 2) grown in 250 ml conical flasks containing 50 ml medium were passed through a nylon sieve with 250 μm pores followed by a sieve with 100 μm pores. The cells which passed through the 100 μm sieve were collected by centrifugation (380×g, 2 min) and inoculated at a final density of 2.5 ml Packed Cell Volume (PCV) per 50 ml of test medium in 250 ml conical flasks. The culture flasks were placed on an orbital shaker (100 rpm) at 25 ± 1 °C under 16 hrs illumination of 30 μmol m^{-2} s^{-1} from cool white fluorescent tubes.

Co-culture system of carrot cells and *E. carotovora* that has been developed in this study is summarised in Figure 2.2 and shown in Plate 3.

1.4.5. Assessment of Cell Viability of Carrot Suspension Culture

Cell viability in the suspension cultures was assessed with fluorescein diacetate (FDA) (Widholm, 1972). A stock solution (0.5% w/v) was made up in acetone and stored at 4 °C. A drop of the stock solution was mixed with a drop of cell suspension on a haemocytometer slide and after about five minutes incubation, the cells were examined under the Olympus BH-2PC fluorescence microscope with the excitation light wavelength in the blue region (530 nm). The fluorescein produced in living cells gave green fluorescence whereas dead cells did not fluoresce (Plate 4). 16 squares in the haemocytometer (0.2 mm deep) were viewed and the viabilities of single cells and cell units were assessed in three areas with two fold replication for each treatment.

Plate 3: **Co-culture of carrot cells and *E. carotovora* subsp. *carotovora* (SCRI 193). Black-filled arrow shows embryogenic cell, black arrow shows ruptured cell, white arrows show bacterial cells and white-filled arrows show non-embryogenic cell (×350)**

Plate 4: **Ten-day-old embryogenic suspension cultures of carrot cv. Morot Duke. Viable cells are stained with FDA (×175)**

Plate 3

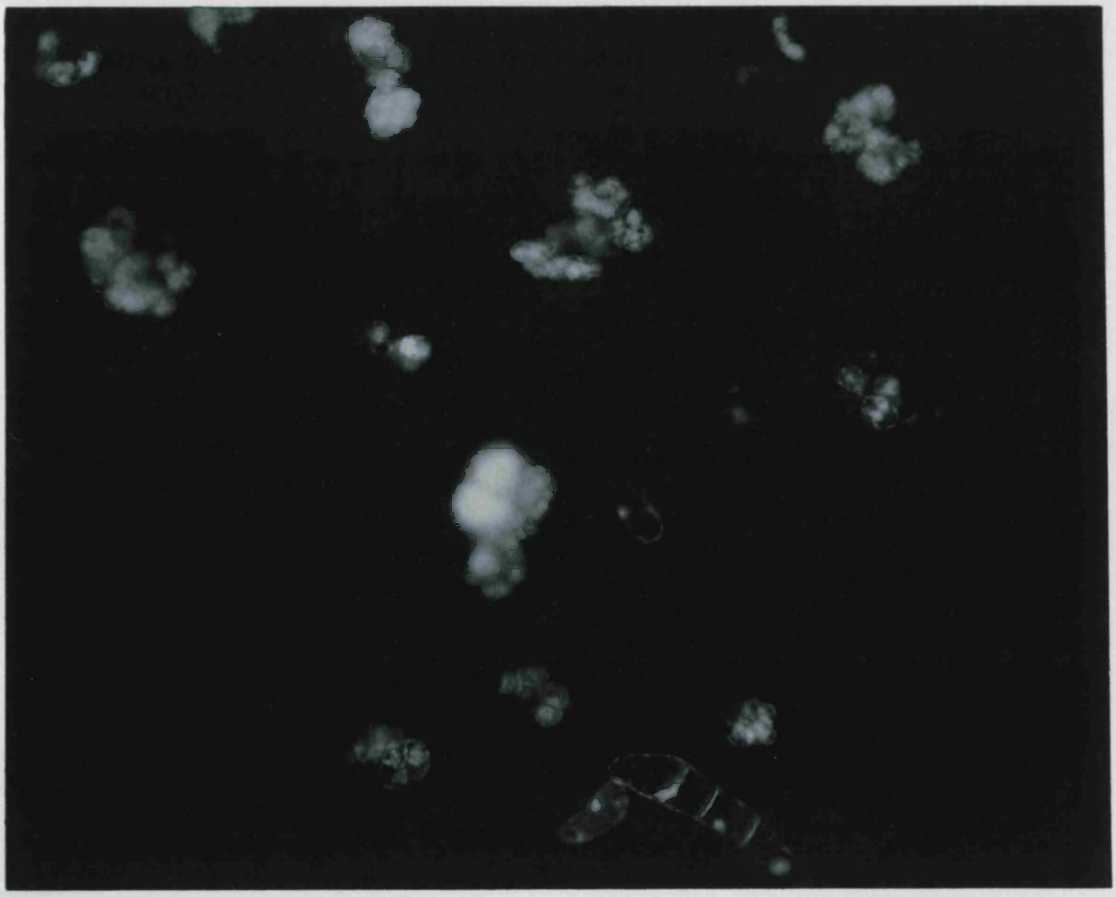


Plate 4

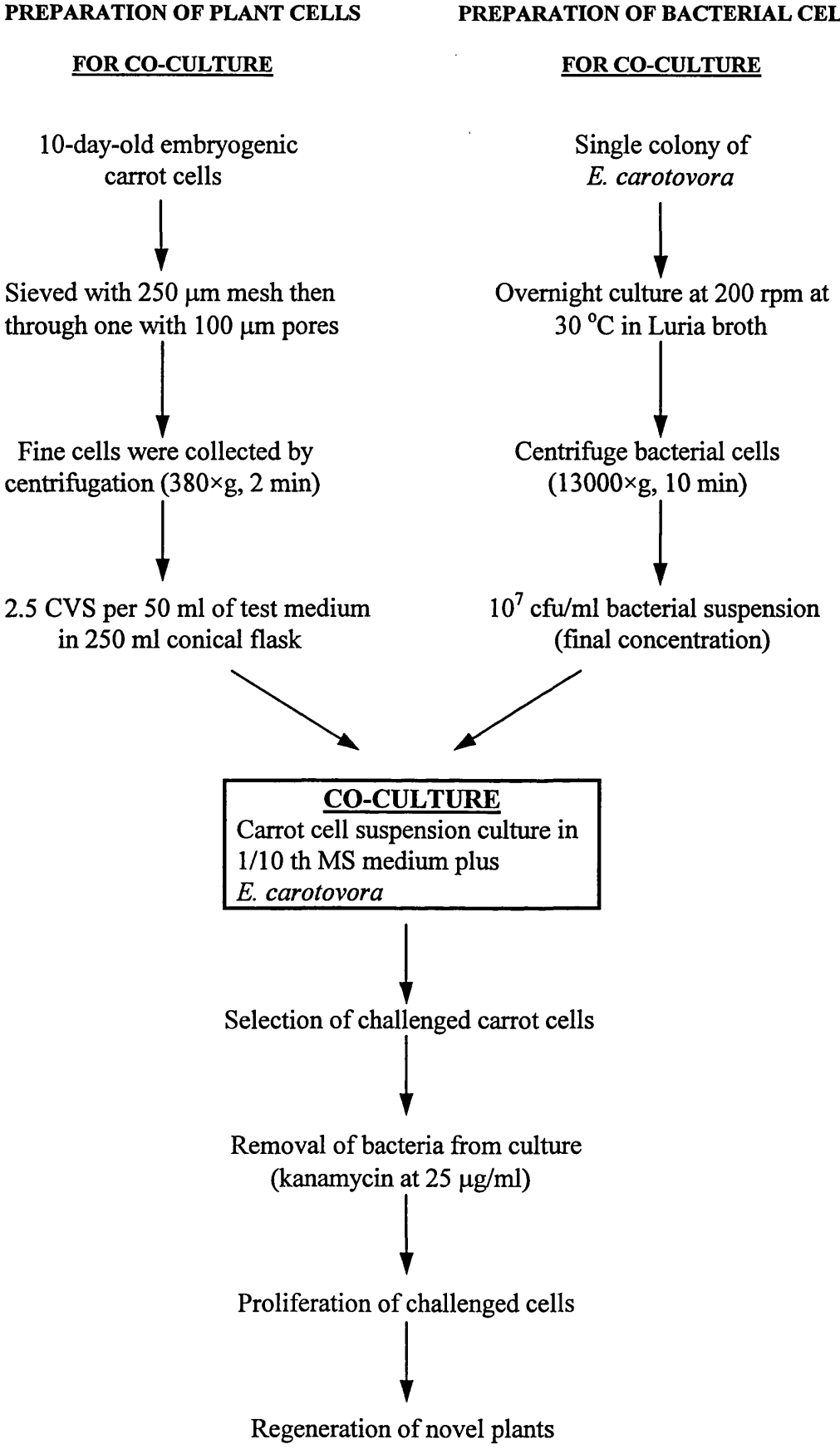


Figure 2.2: Schematic diagram of co-culture of carrot plant cells and *E. carotovora*

For the purpose of estimating the approximate number of viable cells in the cell units, the units were assigned to size categories which were assumed to contain 2, 4, 8, 16, 32 or 48 cells.

1.4.6. Determination of Fresh Weight of Cell Fractions from Suspension Cultures

The fresh weights of the three cell fractions in stock cultures were measured by using a syringe into which had been inserted a sintered polythene disk. Suspensions containing the cell fractions were poured into a weighed syringe and most of the water was removed under vacuum in a Buchner funnel before centrifugation at 700×g for 2 min to remove the remaining surface water from the cells. The syringe was re-weighed in order to determine the fresh weight of the cells.

1.5. ACCLIMATIZATION OF PLANTLETS REGENERATED FROM CALLUS AND SUSPENSION CULTURES

When carrot plantlets derived from the somatic embryos reached sufficient size (4-8 cm in length) they were transplanted into soil. First, the plantlets were transferred to distilled water to avoid dehydration and remove excess adhering medium, then placed in half trays containing Fison F2 compost. They were watered with sterile distilled water and the trays were covered with clear plastic domes to maintain high humidity conditions.

Plantlets were incubated under a 16 hour photoperiod at 25 ± 1 °C for two weeks. The plantlets were acclimatized to growth room humidity conditions by gradually opening the ventilators in the plastic domes and after one week the domes were completely removed. Two weeks later plantlets were transferred to 18 cm deep×13 cm diameter (2 litres capacity) pots containing Fison M2 compost and

grown in a glass house for production of taproots at a minimum temperature of 15-20 °C and 16 hrs daylight.

1.6. PRODUCTION OF SEEDS FROM REGENERATED PLANTS

Since carrot is a biennial plant, the roots were given a cold treatment to induce flowering. The roots were lifted from the pots after removal of the shoots and placed in paper bags in a cold room (4 °C) for 2 months. The roots were then repotted in soil to produce flowering shoots and placed in the green house at 25 ± 1 °C. The viable seeds were collected from the flowers and kept in sealed packets at 4 °C until required.

2. BACTERIAL ISOLATES

Wild types and mutants of *Erwinia carotovora* subsp. *carotovora, Erwinia carotovora* subsp. *atroseptica* and *Erwinia chrysanthemi* supplied from different sources were studied in this research. The characteristics of the bacteria are shown in Table 2.1.

2.1. CULTURE MEDIA AND PREPARATION OF BACTERIAL INOCULUM

The bacterial suspensions were prepared from a single colony into 30 ml sterile universals with 20 ml Luria broth (LB) (Miller, 1972) containing 12 g Tryptone, 5 g Yeast Extract and 5 g NaCl per 1 litre double distilled water (DDW).

Cultures were shaken overnight at 200 rpm at 30 °C. The bacterial number used in the experiments was adjusted to 10^8 cfu/ml spectrophotometrically at 600 nm. The bacterial cells were spun down by centrifugation (11500×g for 1.5 ml tube and 4000×g for 50 ml tube for 10 min) and were replaced with culture media.

Solid media were prepared with Nutrient Agar (NA) by adding 12 g agar (Oxoid) per litre of liquid.

2.2. MAINTENANCE AND STORAGE OF BACTERIAL ISOLATES

One single colony from each bacterial strain was streaked out on NA plates and these plates were incubated for 2 days at 30 °C and then removed to 4 °C for short term storage (3-4 weeks). For long term storage the bacterial suspensions were prepared from a single colony into 30 ml sterile universals with 20 ml LB. 25% (v/v final concentration) sterile glycerol was then added to cultures, mixed thoroughly and frozen in sterilized eppendorfs at -20 °C or -70 °C. The bacterial cultures were thawed at room temperature and streaked out onto NA plates, whenever required.

2.3. MEASUREMENT OF BACTERIAL GROWTH

One single colony from each of two isolates was inoculated into 250 ml conical flasks containing 100 ml LB broth and grown at 30 ± 1 °C on a shaker at 200 rpm. The cell number in growing liquid culture was measured by reading absorbance at 600 nm with a PU 8650 visible spectrophotometer and viable counts were obtained by dilution-plating known volumes (100 µl) of the liquid culture on NA followed by incubation at 30 °C for 48 hrs. Samples were taken at different time intervals and plated onto 3 petri dishes. The change in absorbance at 600 nm of 2 strains of *E. carotovora* in LB broth was observed at 20-30 min intervals until their absorbance reached either 0.8 or 1.0. The mean of three replicated readings for each bacterial strain was plotted with standard deviation.

Figures 2.3 and 2.5 show the standard curve of absorbance at 600 nm versus the number of bacteria per ml of isolates SCRI 1039 and SCRI 193. It took about 6 hrs for *Ecc* and 10 hrs for *Eca* cultures to pass from lag-phase to exponential phase (Figures 2.4 and 2.6).

Table 2.1: The characteristics of bacteria used in the project

NAME	RELEVANT CHARACTERISTICS	SOURCE	
I-*Erwinia carotovora* subsp. *carotovora*			
SCRI 1039	Wild type	Scot. Crops Res. Ins.	M. Pérombelon
SCRI 193	Wild type	Univ. of Warwick	G. Salmond
GS 7000	EMS-induced Cel$^-$	"	"
PR 54	Tn*phoA*-induced Out$^-$	"	"
RJP 116	rex*I*$^-$ (Pel Cel Prt ↓)	"	"
RJP 243	rex$^-$ (non-rex*I*$^-$) (Pel Cel Prt ↓)	"	"
PFP 16	Tn*phoA*-induced hyper (Pel Cel Prt ↑)	"	"
ATTn 5	Carbapenem producer Tn5-induced RM mutant	"	"
MS 10	Lac$^-$ *carI*$^-$ from ATTn5	"	"
AC 5010	Out::Tn5 (Km^r)	Univ. of Missouri Colombia	A. Chatterjee
AC 5013	Out::Tn5 (Tc^r)	"	"
AC 5017	Out::Tn10-lacZ Tc^r)	"	"
AC 5031	aep A-lacZ, Km^r	"	"
***Ecc* 71**	Wild type	"	"
II-*Erwinia carotovora* subsp. *atroseptica*			
SCRI 139	Wild type	Scot. Crops Res. Ins.	M. Pérombelon
SCRI 1043	Wild type	Univ. of Warwick	G. Salmond
94.15	Tn5-induced reduced virulence mutant *mopE*$^-$	"	"
III-*Erwinia chrysanthemi*			
UM 1004	PelAE$^-$ Km^r	Univ. of Cornell	A. Collmer
CUCPB 5019	PelABC$^-$ Km^r	"	"
CUCPB 5018	PelBCE$^-$ Km^r	"	"
UM 1005	PelABCE$^-$ Km^r	"	"
AC 4150	Wild Type	"	"

Out$^-$: non-secretory mutant **rex*I*:** exoenzyme regulation inducer mutant **Prt:** protease **non-rex*I*$^-$:** non-exoenzyme regulation inducer mutant **Cel:** cellulase **rex$^-$:** down regulated enzyme mutant **Pel:** Pectate lyase (genes A-E) **Km^r:** kanamycin resistant **Tc^r:** tetracycline resistant **Tn:** transposon **EMS**: Ethylmethanesulphonate **aep A:** activator of extracellular protein production ***mopE*$^-$:** motility and pathogenicity deficient mutant **RM:** 3D restriction modification system. ***carI*$^-$:**down regulated carbapenem inducer mutant

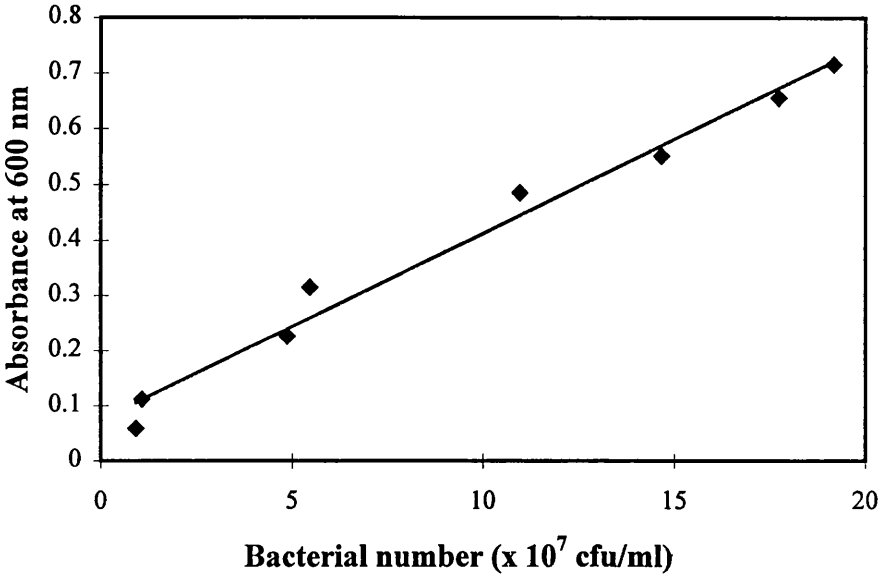


Figure 2.3: Standard curve of absorbance at 600 nm versus bacterial number of *Ecc* isolate SCRI 1039

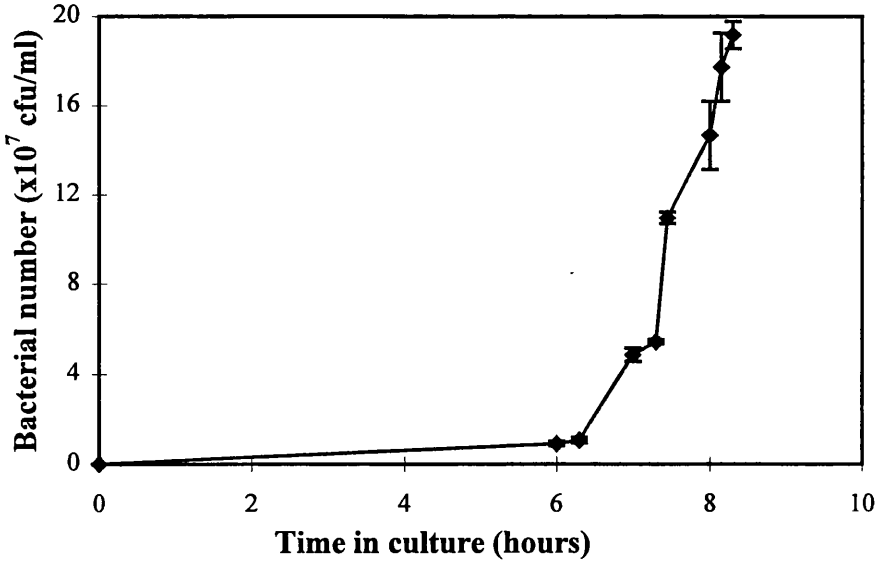


Figure 2.4: Growth rate of *Ecc* isolate SCRI 1039 (viable count)

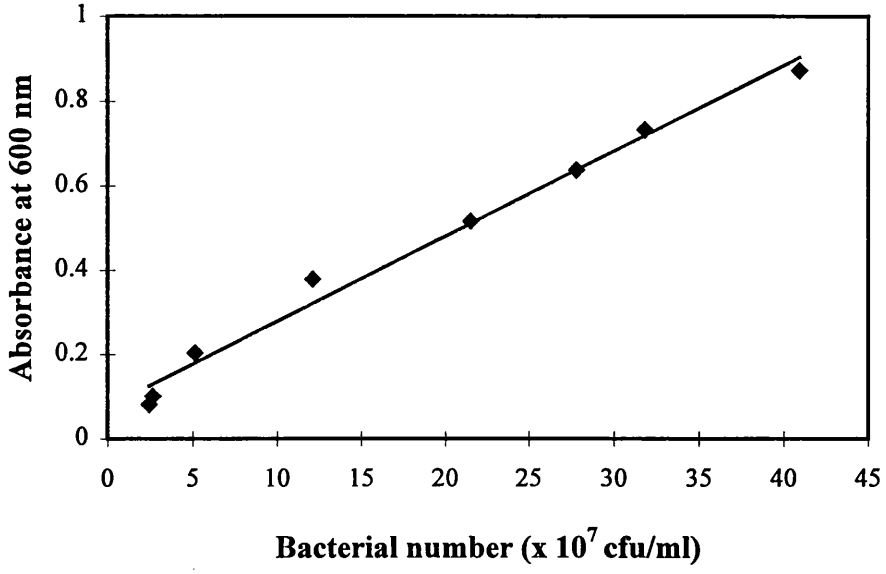


Figure 2.5: Standard curve of absorbance at 600 nm versus bacterial number of *Eca* isolate SCRI 139

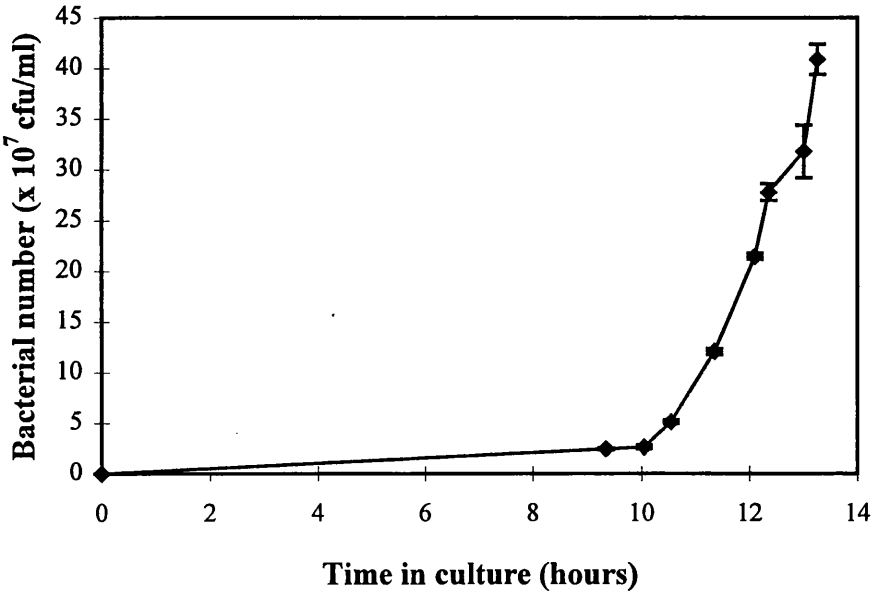


Figure 2.6: Growth rate of *Eca* isolate SCRI 139 (viable count)

3. INOCULATION OF CARROT TAPROOTS WITH *E. carotovora*

Carrots supplied locally (cultivars unknown) were washed, sterilized in sodium hypochlorite (1% available Cl^{-}) for 10 minutes, rinsed in distilled water and left to air dry in a flow cabinet for 2-3 hrs.

An overnight bacterial suspension was diluted with sterile distilled water. A cork borer (No. 2) was used to make wounds at different site along carrot tap roots. Sterile disposable pipette tips (200 µl) were discharged from wounds after 20 µl bacterial suspension at different concentrations was inserted. Sterile distilled water was used as control. Wounds were sealed with Vaseline and tap roots were placed into sandwich boxes containing moist tissue. These boxes were incubated in the dark at 22 ± 1 °C and 98% RH in a growth chamber.

4. *Pythium violae* ISOLATE

The isolate of *Pythium violae* was obtained from Dr. G. White, Horticulture Research International, Wellesbourne. It was isolated from typical cavity spot lesions and is used routinely at HRI for inoculating carrot cultivars.

4.1. MAINTENANCE OF *P. violae*

The isolate was maintained for short term storage on V8 juice agar, containing 200 ml V8 vegetable juice and 20 g agar per litre of V8 juice agar. pH of the medium was adjusted to 6.0 with 1 M NaOH and sterilized at 121 °C, 1.4 bar for 15 min.

The isolate was maintained for long term storage on water agar (WA) plates supplemented with 1.5% (w/v) agar in litre and stored at 14 °C in incubator. The stock cultures were subcultured at 3-month intervals. Whenever required, 0.5 cm^2 square plugs cut from stock water agar cultures of *P. violae* were inoculated onto freshly

prepared V8 or Corn Meal Agar (CMA) plates. Inoculated plates were stored at 20 ± 1 °C and were ready to use after 7 days when the growth of the fungus reached the edge of the plates. Bacterial contamination of the stock cultures was prevented by the addition of 30 μg/ml rifamycin into the media.

4.2. INOCULATION OF CARROT TAPROOTS WITH *P. violae*

Taproots of cv. Burton obtained from Mortimer's farm, Bromham, Wilts, cv. Morot Duke taproots from Bathampton Field Station (University of Bath) or taproots from an unknown cultivar supplied freshly from a local farm were studied. Inoculation was conducted on the same day as roots were harvested. Carrots were gently washed, with care being taken not to damage the periderm.

The roots were placed in plastic boxes lined with two layers of damp tissue and inoculated on their surfaces with 5 mm diameter disks (No. 2 cork borer) taken from freshly growing edge of CMA or V8 cultures of *P. violae*. *Pythium violae* infested seeds were also used as an alternative inoculum (Chapter 3.II, Section 3.4). The roots were incubated in the dark for 7-10 days in 90% RH and at 20 ± 3 °C. Five days after inoculation the plugs were removed and cavity spot formation was assessed by measuring the diameter of lesions and percentage of sites that resulted in infection.

5. THE SOURCE AND MAINTENANCE OF *Erysiphe heraclei* ISOLATES

Carrot cultivars with claimed different levels of resistance to powdery mildew as explained in Section 1.1 were used. The powdery mildew was maintained on seedlings of the commercial cultivar, Autumn King. The seeds were sown in half trays filled with Fison M2 compost and germinated in a green house at 25 ± 2 °C in

natural daylight. Two-month-old carrot plants were transferred into a green house between rows of already infected carrot plants. Powdery mildew conidia were initially brushed off of the infected leaves onto new plants but also natural spread on air currents ensured continuous infection occurred.

5.1. INOCULATION OF CARROT LEAVES WITH *Erysiphe heraclei*

For experimental work, conidia were brushed off of infected plants 12-24 hrs before inoculation. This allowed a new flush of conidia to be produced and thereby provided inoculum of relatively uniform age. Seedlings for inoculation were placed at the base of a rectangular spore settling tower and inoculated by putting the infected carrot leaves over the upper end of the tower and tapping off the conidia. By this method conidia were evenly distributed over the leaf surface. The inoculated carrot seedlings were placed in a green house at 25 ± 2 °C in natural daylight. The inoculum density was monitored microscopically by placing a Vaseline-coated microscopic slide among the plants.

6. ENZYME ASSAYS

6.1. PECTATE LYASE (PGL) AND PECTIN LYASE (PL)

Pectate lyase and pectin lyase activities were assayed spectrophotometrically (Cecil UV-C=2040 [2000 series] spectrophotometer) by measuring the production of 4,5-unsaturated galacturonides with an absorption at 235 nm and 240 nm respectively (Dow et al., 1987). Reaction mixture for PGL assay contained 0.7 ml 0.25% (w/v) sodium polypectate (NaPP) (Sigma Chemical Co., Ltd.) in 0.05 M Tris-HCl buffer (pH 9.0), 0.001 M $CaCl_2$ and 0.3 ml culture supernatant in a total volume of 1 ml. PL assay mixture was as for PGL but with pectin (Sigma) as substrate and 0.01 M $CaCl_2$·

The rate of reaction was measured directly after adding enzyme into a quartz cuvette. The control used in the assay was autoclaved heat-inactivated culture supernatants.

Reaction mixtures were incubated at 30 °C and the increase in absorbance, relative to a substrate blank, was determined at 5 and 10 minutes intervals. One nanokatal (nkat) of enzyme forms 1 nmole of 4,5-unsaturated product per second under the conditions of the assay. The molar extinction coefficient of the unsaturated compounds from pectate and pectin (4600 M^{-1} cm^{-1} at 235 nm and 5500 M^{-1} cm^{-1} at 240 nm respectively) allowed conversion of optical density values into product (Collmer et al., 1988).

6.2. ENDO-POLYGALACTURONASE (endo-PG)

Endo-polygalacturonase activity was determined by viscometry at 25 ± 1 °C. 8 ml of substrate solution (4% [w/v] polygalacturonic acid sodium salt supplied from Sigma) buffered at pH 5 with 0.1 M citrate buffer and 2 ml of enzyme solution were used. Technico viscometers, size 200 ml in which (10 ml) flow rate of water was *ca.* 10 seconds were used. Endo-PG activities were expressed as relative viscometric units (RVU), defined as 10^3 multiplied by the reciprocal of time (min) for a 25% decrease in relative viscosity of substrate (Cooper and Wood, 1975).

6.3. ESTERASE (SUBERINASE)

Esterase activity was measured in reaction mixtures containing 1.6 ml of 0.1 M phosphate buffer pH 8.0, 0.2 ml of Triton X-100 solution (2 g per 500 ml water), 0.2 ml of enzyme solution and 1 ml of *p*-nitrophenol butyrate, incubated at 30 °C for 30 min (Kolattukudy et al., 1981). The substrate was prepared by dissolving 21 mg of *p*-nitrophenol butyrate and 160 mg of Triton X-100 into a 100 ml beaker with ethyl

ether. The solvent was evaporated off with a steam of nitrogen; 50 ml of water was then added and the beaker was heated on a steam bath until the substrate formed a liquid layer. The mixture was then treated with an ultrasonic probe for 1 min at 40% maximum power. Upon cooling, a clear solution was obtained which was used in the assay. Absorbtion was read at 405 nm (Shimadzu UV-260 spectrophotometer). By using the molar extinction coefficient of *p*-nitrophenol (18500 M^{-1} cm^{-1} at 405 nm and pH 8.5), the optical density values were converted into nkat (Fernando et al., 1984).

6.4. CELLULASE (endo-GLUCANASE)

Cellulase activity was measured by viscometry as with PG assay (Cooper and Wood, 1975). Two ml enzyme solution was placed in Technico viscometers (size 200) at 25 ± 1 °C and mixed with 8 ml, 0.7% (w/v) carboxymethylcellulose (CMC) (British Drug House, [BDH]) in 0.1 M citrate buffer (pH 5.0). Cellulase activity is expressed as relative viscometric units (RVU) as defined in Section 6.2.

7. SUBERIN EXTRACTION

Suberin was prepared from potato periderm, according to the method of Fernando et al. (1984). Potato peels were boiled in water to separate the periderm. After being washed thoroughly with water, the periderm was boiled in a solution of ammonium oxalate (16 g/l), oxalic acid (4 g/l) to remove adhering internal tissue. The periderm was separated by filtration and washed with water before being treated with cellulase, (Sigma, 1g per 500 ml), amylase (Sigma, 0.1 g per 500 ml) and macerozyme (0.5 g/l) in 0.05 M sodium acetate buffer, pH 5.0 at 30 °C for 16 hrs with stirring. The remaining residue was dried and subjected to Soxhlet extraction with chloroform for 24 hrs (72 refluxes). The final residue was dried, powdered in a mill and stored desiccated.

8. EXTRACTION OF ENZYMES FROM CARROT TISSUES

Tissues collected from infected and uninfected lesions were placed into precooled (-20 °C) mortars and ground to a fine powder in the presence of liquid nitrogen. Tissue was then extracted in 0.025 M sodium phosphate buffer (pH 6) supplemented with 0.02 M NaCl (to desorb enzyme from cell walls), 5 mM dithiothreitol (to prevent oxidation) and 5% (w/v) insoluble polyvinyl polypyrrolidone (PVP, BDH) (to adsorb phenols) at 1 g tissue per 5-8 ml buffer. The ground tissue was stirred in this medium for 15 min at 4 °C and filtered through muslin and clarified by centrifugation at 20000×g for 15 min. The extract was dialysed overnight in distilled water (pH 7) at 4 °C and concentrated by addition of polyethylene glycol (PEG Mr 20,000) (Cooper and Wood, 1980). The liquid extracts were stored at -70 °C until required for use in the enzyme assays.

9. EXTRACTION OF CARROT CELL WALLS

Carrots (cultivar unknown) were obtained locally. Ends of taproots were removed and the remainder chopped coarsely into thin discs. The tissues were then comminuted in a Warring blender with cold 0.1 M phosphate buffer pH 7.0 at 4 °C. The debris was filtered using double folded muslin, resuspended in the buffer and the process repeated. The resulting residue was resuspended and filtered twice using cold distilled water. As much liquid as possible was squeezed out of the residue and the insoluble material was resuspended and filtered through muslin twice in 2 litres of chloroform: methanol (1:1). It was then resuspended in excess acetone and filtered twice. The carrot cell walls were placed in glass petri dishes with the lids slightly off to dry for 12 hours in a fume extraction hood. These petri dishes were sealed and stored in a desiccator.

10. SCANNING ELECTRON MICROSCOPY

Specimens of carrot leaves were mounted on an aluminium disc attached with a mixture of tissue tek (O.T.C. Compound, Miles Inc. USA) and carbon dag (Colloidal graphite, Agar Scientific UK) (50:50) and rapidly frozen in liquid nitrogen slush transferred via the cryo preparation chamber (Oxford Instruments, [Oxford, UK] Cryo Preparation System model CT 1500) to the scanning electron microscope where frost was sublimed from the surface. They were then returned to the cryo preparation chamber and sputter-coated with gold to a thickness of about 20 nm. Specimens were then examined with a JEOL, (Tokyo, Japan) JSM 6310 scanning electron microscope at either 5, 10 or 15 kV.

11. STATISTICAL ANALYSIS

All analyses were computed with Minitab (Minitab Inc., USA) and Microsoft Excel Version 5 statistical programmes. The completely randomised design was used for numerical data whereas enumerical data were analysed using Chi-Square. The means were separated by Duncan's Multiple Range Test at 5% level. The viability of plant cells was shown as percentage and these values were converted into angular transformation before analysis of data.

CHAPTER 3

RESULTS AND DISCUSSION

I - SOMATIC EMBRYOGENESIS IN CARROT

1. INTRODUCTION

Embryogenic callus and suspension cultures are widely used for obtaining transgenic plants, somaclonal variants and mutants (Chapter 1). Callus cultures usually provide the material from which cell suspension cultures are derived; and in addition they present a convenient form for the maintenance of cell lines and are the type of culture in which plant regeneration is usually initiated. In this study embryogenic callus cultures of carrot were studied to screen regenerants to select for resistance to obligate parasite *E. heraclei*. Carrot cell suspension cultures have been used to generate large amounts of cell material, to provide relatively homogeneous cell populations and to examine growth quantitatively as well as to select and regenerate novel disease resistant plants following challenge with pathogens.

Somatic embryos were first observed in carrot suspension cultures by Steward et al. (1958) and in callus cultures on semi-solid medium by Reinert (1959). Since then, carrot has become a model system for the study of somatic embryogenesis. The

techniques for growth of carrot callus and suspension culture systems are therefore well established and it is known that the type of explant, its size, age and the manner in which it is cultured, can all affect whether the cultures can be successfully initiated and whether morphogenesis can be induced. In order to exploit the potential for somatic embryogenesis in this investigation it was necessary to develop the optimum techniques for the initiation and maintenance of suspension and callus cultures. It was also necessary to establish fine embryogenic suspension cultures with high potential for regeneration of plantlets for use in the co-culture experiments described in Chapter 3.III to select disease resistant or tolerant plants.

2. INDUCTION OF SOMATIC EMBRYOGENESIS IN EXPLANTS OF CARROT ON SEMI-SOLID MEDIUM

2.1. OPTIMISATION OF SOMATIC EMBRYO PRODUCTION FROM COTYLEDON AND HYPOCOTYLS OF CV. NRI-92

A number of factors were investigated with semi-solid media to establish an optimum procedure for the production of somatic embryos from the hypocotyl and cotyledons of the carrot cultivar NRI-92. These included the effects of light on the seedlings from which the explants were taken, the age of the seedlings, the type of explant, the effect of wounding and the effect of activated charcoal in the embryo development medium.

The surface sterilisation of carrot seeds was performed as explained in Chapter 2, Section 1.2. Seedlings of different ages, grown either in the light or in the dark, were used as the sources of explants. Dark conditions were created by wrapping with aluminium foil. As germination of the seeds was highly asynchronous, the physiological age of seedlings was assessed according to the length of their hypocotyl.

Cotyledon explants were taken from seedlings of different ages characterised by their hypocotyl lengths and kept in three separate groups according to the hypocotyl length. Hypocotyl explants (1-3 mm, 4-6 mm, 8-10 mm in length respectively) were also taken from these groups of seedlings.

One of the cotyledons from each seedling was wounded on 3 to 5 sites with a dissecting needle whereas the other was left undamaged and in addition each cotyledon was divided transversely into two halves. The four pieces of cotyledon thus obtained from each seedling were placed into the same petri dish and explants from 12 seedlings were cultured on "embryo induction medium" (MS2 medium supplemented with 5 μM 2,4-D).

Callus initiated on the MS2 medium supplemented with 5 μM 2,4-D was transferred to "embryo development medium" (either MS2 or to MS2 + 1% w/v activated charcoal medium [AC]) after two weeks. After three weeks, the embryogenic response of the tissue was assessed and the presence of embryogenic tissue (including globular, heart and torpedo stage somatic embryos) was scored (Plate 5B, 5C). 100% of callus pieces had some embryogenic tissue.

The percentage of callus pieces producing embryogenic structures was scored, together with an estimate of the amount of embryo production in each callus piece according to the following scale:

0: callus did not produce embryogenic tissue

1: up to 25% of the area of the callus piece produced embryogenic tissue

2: 26-50% of the area of the callus piece produced embryogenic tissue

3: 51-75% of the area of the callus piece produced embryogenic tissue

4: 76-100% of the area of the callus piece produced embryogenic tissue

The results obtained with the cotyledon explants are presented in Table 3.1 and those obtained with the hypocotyl explants are presented in Table 3.2. Data which were obtained from an average of somatic embryo production per callus were subjected to an analysis of variance test followed by Duncan's Multiple Range Test (5%) using a completely randomised design to separate the means where significance was indicated.

2.1.1. Somatic Embryo Production from Cotyledon Explants

Since the analysis of variance (Appendix 2) shows significant differences ($p < 0.01$) between the effects of four of the treatments (types of explants, ages of seedlings, wounding and seedling light treatments) and no significant differences between any of the interactions, the results obtained with these particular treatments are combined in tables presented in Appendix 3. It can be seen from Tables 3.1 and 3.2 that the highest production of somatic embryos, both in terms of somatic embryo production and the proportion of responsive explants, was obtained with the basal parts of wounded cotyledon explants which had been excised from seedlings with the shortest hypocotyls (1-3 mm) and which had been grown in the dark.

2.1.2. Somatic Embryo Production from Hypocotyl Explants

The analysis of variance (see Appendix 4) relating to the results in Table 3.2, indicates that there are no significant differences between the effects of any of the treatments on the hypocotyl of different ages. The data, however, show that both the highest embryogenic response and highest somatic embryogenic production per explant consistently occurred with the 4-6 mm hypocotyl explants regardless of the other treatments.

Table 3.1: The effects of seedling age, type of explant, wounding, light, and media on induction of somatic embryogenesis on carrot cv. NRI-92 cotyledon explants

Light condition for seedlings	Wounding*	Embryo development media	Length of hypocotyl (mm)	Type of cotyledon explant	Embryogenic response (%)	Mean embryogenic tissue score per treatment**
Light	Wounded	MS2 + AC	1-3	Top	67	1.4
				Base	83	2.1
			4-6	Top	50	0.8
				Base	92	1.3
			8-10	Top	33	0.3
				Base	58	0.9
		MS2	1-3	Top	70	1.2
				Base	80	2.0
			4-6	Top	17	0.2
				Base	58	0.8
			8-10	Top	17	0.2
				Base	25	0.4
	Intact	MS2 + AC	1-3	Top	50	1.6
				Base	67	1.3
			4-6	Top	33	0.3
				Base	58	0.2
			8-10	Top	25	0.3
				Base	58	0.4
		MS2	1-3	Top	30	0.5
				Base	80	1.6
			4-6	Top	0	0
				Base	42	0.6
			8-10	Top	33	0.3
				Base	25	0.6
Dark	Wounded	MS2 + AC	1-3	Top	67	1.7
				Base	92	2.5
			4-6	Top	50	0.4
				Base	80	1.4
			8-10	Top	50	0.3
				Base	75	0.8
		MS2	1-3	Top	67	1.2
				Base	92	1.8
			4-6	Top	50	1.0
				Base	80	1.6
			8-10	Top	50	0.8
				Base	75	1.6
	Intact	MS2 + AC	1-3	Top	67	1.1
				Base	58	1.6
			4-6	Top	25	0.3
				Base	17	1.0
			8-10	Top	25	0.4
				Base	33	0.5
		MS2	1-3	Top	58	0.6
				Base	75	1.8
			4-6	Top	20	0.4
				Base	80	1.1
			8-10	Top	9	0.1
				Base	50	0.6

* : Wounding applied to cotyledon explants. ** : scoring system (see Section 2.1). Means were calculated from 12 replicated samples. **AC:** Activated charcoal (1% w/v). Explants were cultured on MS2 supplemented with 5 μM 2,4-D for two weeks prior to transfer to embryo development medium and data were scored after 3 weeks in embryo development medium.

Table 3.2: The effects of length of hypocotyl, light and media on induction of somatic embryogenesis on carrot cv. NRI-92 hypocotyl explants

Light conditions for seedlings	Embryo development medium	Length of hypocotyl explant (mm)	Embryogenic response (%)	Mean embryogenic tissue score per treatment*
Light	MS2 + AC	1-3	**25**	**0.3**
		4-6	**67**	**1.4**
		8-10	**50**	**0.8**
	MS2	1-3	**60**	**1.6**
		4-6	**75**	**2.0**
		8-10	**25**	**0.3**
Dark	MS2 + AC	1-3	**50**	**0.8**
		4-6	**75**	**1.3**
		8-10	**50**	**0.8**
	MS2	1-3	**50**	**1.3**
		4-6	**60**	**1.8**
		8-10	**58**	**0.8**

* : scoring system (see Section 2.1). Means were calculated from 12 replicated samples. **AC:** Activated charcoal. Explants were cultured on MS2 supplemented with 5 µM 2,4-D for two weeks prior to transfer to embryo development medium. Data were scored after 3 weeks in embryo development medium.

2.2. THE EFFECTS OF EXPLANT TYPE, BASAL MEDIA AND CONCENTRATION OF 2,4-D ON SOMATIC EMBRYOGENESIS FROM HYPOCOTYL AND COTYLEDON EXPLANTS OF CV. NRI-92

Callus cultures were initiated from carrot cv. NRI-92 hypocotyl explants (4-6 mm in length) and entire cotyledon explants from the same seedlings. The effects of two basal media were examined: modified Lin and Staba (LS) (1961) as described by Fujimura and Komamine (1979a) and Murashige and Skoog (MS) (1962), supplemented with four different concentrations of 2,4-D (0.05 µM, 0.5 µM, 5 µM and 50 µM) (see Appendix 35). Five petri dishes with 4 explants per dish were cultured for one week. The explants were then subcultured onto each corresponding medium without 2,4-D and the data recorded after a further three weeks. The results are presented in Table 3.3.

Table 3.3: The effects of 2,4-D concentration, basal media and explant on somatic embryogenesis of carrot cv. NRI-92

Basal Medium	2,4-D (μM) in embryo induction medium	Embryogenic response (%)		Mean embryogenic tissue score per treatment*	
		Cotyledon	Hypocotyl	Cotyledon	Hypocotyl
MS	0.05	53	19	0.6	0.2
	0.5	53	40	0.6	0.5
	5	61	50	1.1	1.2
	50	23	55	0.3	1.0
LS	0.05	35	15	0.4	0.2
	0.5	43	35	0.6	0.6
	5	15	55	0.2	0.9
	50	70	25	0.8	0.4

* : scoring system (see Section 2.1). Means were calculated from 20 replicated samples. Hypocotyl explants, 4-6 mm in length and cotyledon explants from the same seedlings were cultured on MS2 supplemented with various 2,4-D concentrations for one week prior to transfer to each corresponding embryo development medium without 2,4-D. Data were scored 3 weeks after transferred into embryo development medium.

The analysis of variance confirmed that in terms of the effects on somatic embryo production there were no significant differences between the cotyledon and hypocotyl explants, the different concentrations of 2,4-D and the two basal media (see Appendix 5). Nevertheless, MS2 medium generally appeared to be more effective on both cotyledon and hypocotyl explants for the somatic embryo production and embryogenic response. Also, MS2 medium supported better plantlet formation from the somatic embryos than the LS2 medium which was ineffective after a further four weeks (a total of 8 weeks on the MS2 medium. Results are not shown). Among the four 2,4-D concentrations tested, 5 μM 2,4-D tended to produce slightly more somatic embryos than the other concentrations. As can be seen in Table 3.3 the amount of embryogenic tissue produced shows some correlation with the embryogenic response in both types of explant. Although average somatic embryo production obtained from hypocotyl explants

was slightly higher than from cotyledon explants, the embryogenic response of hypocotyl explants was mainly lower than that of cotyledon explants. In conclusion, therefore, hypocotyl explants together with induction treatment for one week on MS2 supplemented with 5 μM 2,4-D resulted in the best production of embryogenic callus.

2.3. THE EFFECT OF TIME OF EXPOSURE TO THE EMBRYO INDUCTION MEDIUM ON SOMATIC EMBRYOGENESIS FROM HYPOCOTYL EXPLANTS OF CV. NRI-92

Carrot cv. NRI-92 was used to determine the optimal time of exposure of explants to the 2,4-D-containing medium for the induction of embryogenic callus and somatic embryo production. Hypocotyl explants, 4-6 mm in length, were cultured on MS2 medium supplemented with two concentrations of 2,4-D (0.5 μM, 5 μM). The cultures were incubated for one, two or three weeks before they were transferred onto MS2 medium for a further three weeks. Twenty eight explants were cultured for each treatment and the final data were recorded after the second culture period (three weeks). The results are presented in Table 3.4.

Table 3.4: The effects of time of exposure to embryo induction medium containing different concentration of 2,4-D on embryogenic response of carrot cv. NRI-92

2,4-D (μM) in embryo induction medium	Time in embryo induction media (days)	Embryogenic response (%)	Mean embryogenic tissue score per treatment*
0.5	7	**29**	**0.7**
	14	**75**	**1.4**
	21	**75**	**1.3**
5	7	**50**	**1.2**
	14	**50**	**1.0**
	21	**25**	**0.8**

* : scoring system (see Section 2.1). Means were calculated from 28 replicated samples. Hypocotyl explants, 4-6 mm in length were cultured on MS2 supplemented with various 2,4-D concentrations prior to transfer to embryo development medium without 2,4-D. Data were scored after 3 weeks in embryo development medium.

As can be seen in Table 3.4, the highest values for somatic embryo production were obtained with exposure to the embryo induction medium containing 0.5 μM 2,4-D for either 14 days or 21 days, although the differences between the various treatments were not statistically significant (see Appendix 6). The highest embryogenic response and somatic embryo production per explant obtained with one week's incubation occurred in the medium containing 5 μM 2,4-D, while with two weeks' incubation the lower concentration of 0.5 μM 2,4-D appeared to be more effective.

In conclusion, either 7 days for incubation at the embryo induction stage on MS2 medium containing 5 μM 2,4-D or 14 days for incubation at the embryo induction stage on MS2 medium supplemented with 0.5 μM 2,4-D could be chosen for efficient somatic embryo production.

2.4. THE EFFECTS OF 2,4-D CONCENTRATIONS AND TYPES OF EXPLANT ON THE SOMATIC EMBRYO PRODUCTION WITH DIFFERENT CARROT CULTIVARS

Three F1 hybrids of carrot cvs.; Morot Duke, Morot Ingot, Morot Favor and the open-pollinated commercial cultivar NRI-92 were screened for their embryogenic competence.

Hypocotyl explants, 4-6 mm in length and cotyledon explants taken from the same seedlings were placed on MS2 medium supplemented with four different concentrations of 2,4-D (0.05 μM, 0.5 μM, 5 μM and 50 μM). Twenty explants were established for each treatment and they were transferred to MS2 medium after incubation for one week on the above media. Results were recorded after three weeks incubation in MS2 medium and are given in Table 3.5.

The highest embryogenic responses (90%) and somatic embryo production were obtained from Morot Duke and Morot Favor (see Table 3.5). The hypocotyl explants were generally superior to the cotyledon explants in terms of the somatic embryo production according to Duncan's Multiple Range Test (5%).

Table 3.5: The effects of 2,4-D concentration and types of explant on somatic embryogenesis with four carrot cultivars

Cultivars	2,4-D (μM) in embryo induction medium	Embryogenic response (%)		Mean embryogenic tissue score per treatment*	
		Cotyledon	Hypocotyl	Cotyledon	Hypocotyl
Morot Duke	0.05	**40**	**44**	**0.6**	**0.7**
	0.5	**63**	**90**	**0.7**	**2.2**
	5	**58**	**80**	**0.9**	**2.4**
	50	**55**	**90**	**1.1**	**2.6**
Morot Favor	0.05	**50**	**35**	**0.5**	**0.6**
	0.5	**80**	**95**	**1.1**	**1.9**
	5	**90**	**88**	**1.4**	**2.3**
	50	**63**	**95**	**0.8**	**1.9**
Morot Ingot	0.05	**50**	**20**	**0.5**	**0.2**
	0.5	**60**	**30**	**0.6**	**0.4**
	5	**66**	**75**	**0.9**	**1.6**
	50	**40**	**60**	**0.6**	**1.3**
NRI-92	0.05	**53**	**19**	**0.6**	**0.2**
	0.5	**53**	**40**	**0.6**	**0.5**
	5	**61**	**50**	**1.1**	**1.2**
	50	**23**	**55**	**0.3**	**1.0**

* : scoring system (see Section 2.1). Means were calculated from 20 replicated samples. Explants were cultured on MS2 supplemented with various 2,4-D concentrations for one week prior to transfer to embryo development medium without 2,4-D. Data were scored after 3 weeks in embryo development medium.

Analysis of variance (Appendix 7) shows significant differences ($p < 0.01$) in terms of somatic embryo production between the effects of the different types of explant and between the concentrations of 2,4-D. There are also significant interactions between the 2,4-D concentrations and the types of explant. Re-arrangement of the data in Appendix 8 shows that, overall, hypocotyl explants from the cultivars Morot Duke and

Morot Favor were the most responsive explants and that the most effective 2,4-D concentration was 5 μM.

It was observed however, that although the callus was much more friable and had generally more embryogenic tissue when cultured on 5 μM 2,4-D, this concentration of 2,4-D inhibited further development of the somatic embryos and few globular and heart shaped somatic embryos reached the torpedo and mature forms in comparison with the lower concentrations (see Section 2.5). In conclusion, therefore, the hypocotyl explant, 0.5 μM 2,4-D and either Morot Duke or Morot Favor could be used in future experiments.

2.5. REGENERATION OF MATURE SOMATIC EMBRYOS FROM EMBRYOGENIC CALLUS CULTURES

This experiment was designed to establish which genotype and which embryo induction medium produced the highest number of mature somatic embryos which were identified as green cotyledonary-stage somatic embryos produced after transfer to the embryo development medium. Fifty 4-6 mm hypocotyl explants were first incubated for two one-month passages on either MS2 medium supplemented with 0.5 μM 2,4-D or MS2 with 5 μM 2,4-D. Ten of the best calluses (in terms of size, green colouration and production of globular embryogenic tissue) from each of the cultivars Morot Duke, Morot Favor, Morot Ingot and Morot Rondino were randomly selected from the responsive explants on each of the embryo induction media and weighed before transfer to the embryo development medium (MS2). The numbers of green cotyledonary stage somatic embryos produced after 4 weeks were recorded.

Table 3.6: The effect of 2,4-D on regeneration of mature somatic embryos from embryogenic callus cultures of four carrot cultivars

	Mean number of regenerated mature embryos per 0.1 g callus	
	2,4-D in embryo induction medium (μM)	
Cultivar	**0.5**	**5**
Morot Rondino	1167 a	73 b
Morot Duke	866 b	187 a
Morot Ingot	802 b	69 b
Morot Favor	692 b	173 a

Hypocotyl explants, 4-6 mm in length were cultured on MS2 supplemented with various 2,4-D concentrations for two months prior to transfer to embryo development medium without 2,4-D. Data were scored after 4 weeks in embryo development medium. Means within columns followed by the same letters are not significantly different according to Duncan's Multiple Range Test (p= 0.05). Means were calculated from 10 replicated samples.

Analysis of variance shows that the effects of cultivar, concentration of 2,4-D and the interactions between cultivars and concentration of 2,4-D had significant effects on the production of cotyledonary-stage somatic embryos from embryogenic callus cultures (see Appendix 9).

Table 3.6 illustrates that with all four cultivars, callus cultures grown on MS2 medium supplemented with 0.5 μM 2,4-D were capable of regenerating significantly more ($p < 0.01$) cotyledonary-stage embryos in comparison with callus cultures grown on MS2 medium supplemented with 5 μM 2,4-D media.

Among the four cultivars used, Morot Rondino produced the highest number of mature somatic embryos at 0.5 μM 2,4-D ($p< 0.01$). There were no significant differences between Morot Duke, Morot Ingot and Morot Favor in terms of mature somatic embryo production according to Duncan's Multiple Range Test (5%) at the lower concentration of 2,4-D.

For efficient production of embryogenic cells and regeneration of mature somatic embryos from the tissue, it can be concluded that MS2 medium supplemented with 0.5 μM 2,4-D at the embryo induction stage, 4-6 mm in length hypocotyl explant and either Morot Duke, Morot Favor or Morot Rondino can be chosen as the standard procedure for future experiments.

3. INDUCTION AND MAINTENANCE OF CARROT EMBRYOGENIC SUSPENSION CULTURES IN LIQUID MEDIUM

3.1. GROWTH RATES OF CELL SUSPENSION CULTURES MEASURED BY CELL VOLUME AFTER SEDIMENTATION (CVS)

As cell suspension cultures usually require more regular subculture than callus cultures, a set of experiments was carried out to establish the optimum subculture times for the growth of embryogenic suspension cultures of carrot. Suspension cultures of Morot Duke, Morot Ingot, Morot Favor and Morot Rondino were initiated from 8-week-old callus induced from hypocotyl explants. Pieces of callus weighing 0.1-0.2 g were transferred into 25 ml liquid MS2 medium supplemented with 0.5 μM 2,4-D in 100 ml conical flasks. A subculture regime was then established whereby 5 ml of the suspension culture was transferred into 45 ml of fresh medium in 250 ml conical flasks.

The content of one flask from each of the four genotypes was subcultured into six conical flasks at 14-day intervals. The growth curves of the suspension cultures were prepared on the basis of the means of the CVSs obtained from the 6 replicate conical flasks.

Figure 3.1 illustrates that the CVS rose steadily in all cultivars, but that the final CVS volumes differed considerably. Morot Rondino increased only 5 times and reached stationary phase by day 12. Likewise, Morot Favor stopped growth by day 12.

In contrast, Morot Duke and Morot Ingot continued to grow by day 14. The highest final CVS values were obtained with the cultures from Morot Duke and Morot Ingot, which apparently had not reached stationary phase by the end of the culture periods. Strictly, a stabilized growth regime can only be established for successive passages if stationary phase is attained but, as this can only be determined on the basis of cell counts and as time was limited, it was assumed for practical purposes that stabilization had more or less been achieved by the end of the 14 day period.

Compared with the other two cultivars Morot Duke and Morot Ingot also produced very fine embryogenic suspensions that could be subcultured easily and which would be most suitable for co-culture with the pathogenic organism. Since the time for this area of research was limited, a single genotype, Morot Duke, was chosen for the co-culture experiments in Chapter 3.III and it was routinely subcultured at 14-day intervals according to the protocol described above.

3.2. VIABILITY OF EMBRYOGENIC SUSPENSION CULTURES OF CV. MOROT DUKE

In addition to use of CVS (see Section 3.1), the development of a subculture regime for the carrot embryogenic suspension cultures was based on measurements of changes in cell viability.

Carrot cv. Morot Duke suspension cultures were prepared as explained in Chapter 2, Section 1.4.4 and three replicate culture flasks were set up. The viability of the cells was evaluated using fluorescein diacetate (FDA) vital stain every 2 days over a period of 16 days (see Chapter 2, Section 1.4.5).

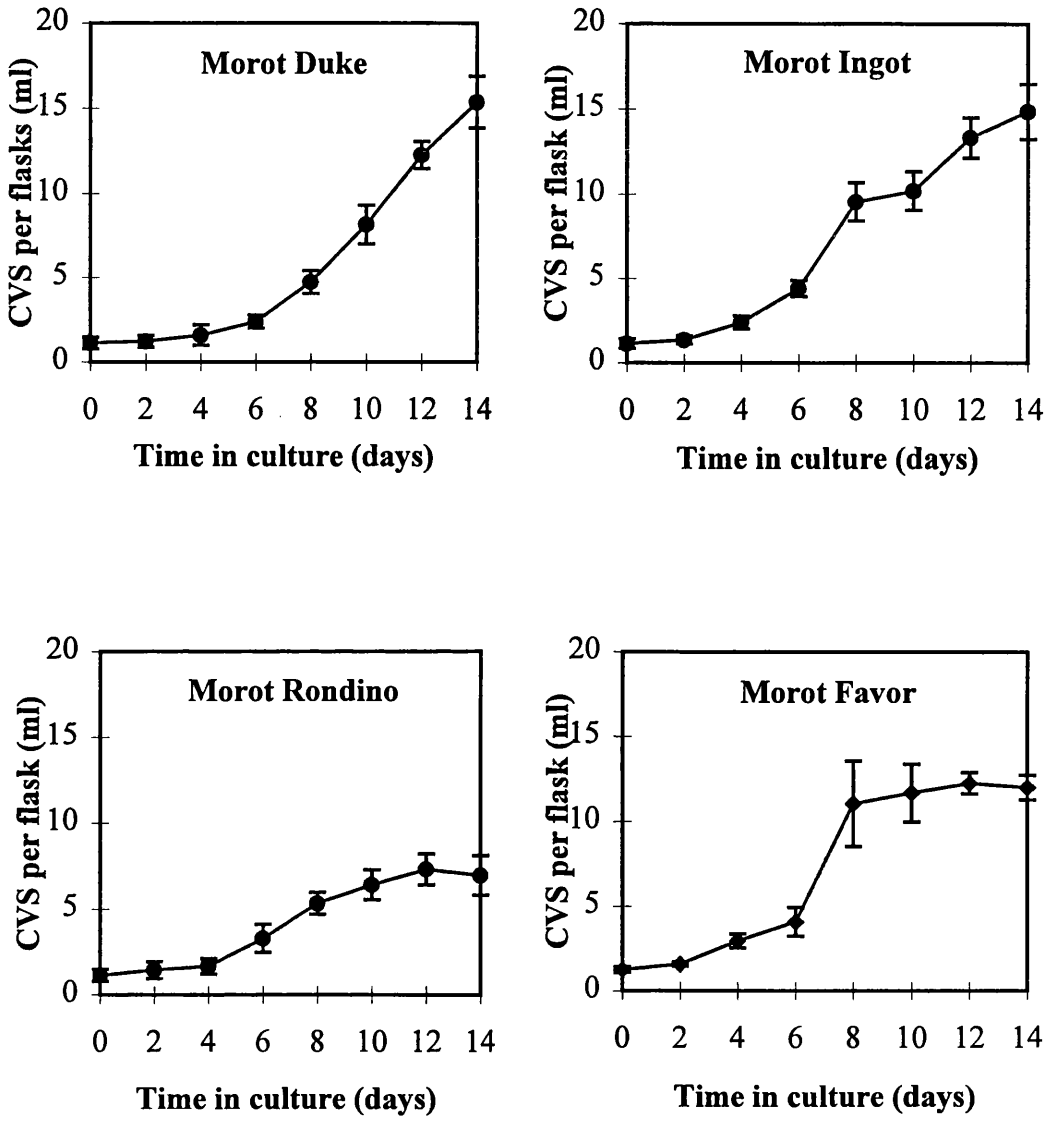


Figure 3.1:Typical growth curves of embryogenic suspensions of carrot cultivars. Means of 6 replicates are presented with standard deviation bars

Cultivars: Morot Duke, Morot Ingot, Morot Rondino, Morot Favor
Medium: MS2 + 0.5 µM 2,4-D
Inoculum: 5 ml into 45 ml fresh medium
Temperature, light and rotation: 25 °C, 30 mol m^{-2} S^{-1} light, 100 rpm
Subculture time: 14 day intervals

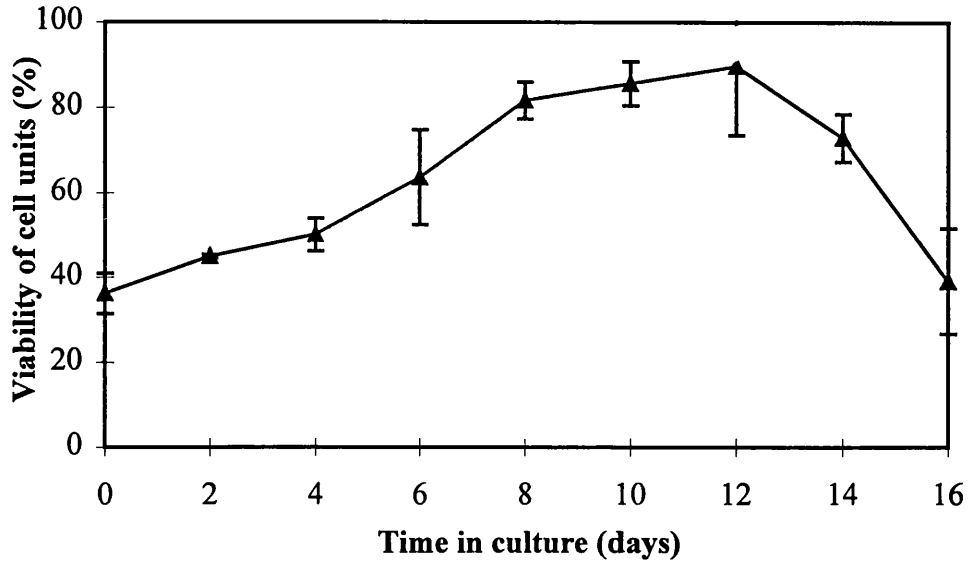


Figure 3.2: Viability of carrot cv. Morot Duke suspension cultures

Data represents the means and standard deviations for 3 replicated flasks.

Figure 3.2 shows that the viability of the suspension cultures increased quite slowly up to a value of 90% at 12 days after which it declined rapidly to approximately the initial value of 36% by day 16. In order to maintain active growth and cell viability, therefore, it was concluded that it would be best to subculture the cells on the basis of this information every 12-14 days.

3.3. THE SIZE DISTRIBUTION OF CELL UNITS IN SUSPENSION CULTURES FROM FOUR CARROT CULTIVARS

Suspension cultures from carrot cvs. Morot Duke, Morot Ingot, Morot Favor and Morot Rondino were examined to determine the effects of subculture regime on the distribution of cell unit sizes since the number of < 100 μm cell units in the cultures would be important with regard to the future co-culture experiments. Five ml taken from two-month-old suspension cultures was subcultured into 45 ml MS2 supplemented with

0.5 μM 2,4-D. At the end of the culture period (16 days) the cells were fractionated to produce three different size ranges (> 250 μm, 100-250 μm, < 100 μm) as described in Chapter 2, Section 1.4.4.

The final fresh weights of the cell fractions were measured with the use of a Buchner funnel (see Chapter 2, Section 1.4.6) over three successive passages, with each on the basis of three replicated flasks. The means of the results from the three passages are shown in Table 3.7.

The results show that carrot cv. Morot Duke produced the highest total fresh weight over the three passages and in all of the cultivars the largest cell units (> 250 μm) formed the greatest proportion of the 16-day-old suspension cultures while the smallest cell units (< 100 μm) were only present in relatively low amounts.

Table 3.7: The fresh weights of different size fractions of cell units from 16-day-old cell suspension cultures of four carrot cultivars

Cultivars	Fraction (μm)	Mean fresh weight per flask (g)	Total fresh weight (g)
Morot Duke	**> 250**	2.37 ± 0.31	3.14
	100 - 250	0.54 ± 0.45	
	< 100	0.23 ± 0.16	
Morot Favor	**> 250**	1.41 ± 0.28	1.78
	100 - 250	0.34 ± 0.13	
	< 100	0.03 ± 0.02	
Morot Ingot	**> 250**	1.39 ± 0.07	2.20
	100 - 250	0.61 ± 0.26	
	< 100	0.2 ± 0.17	
Morot Rondino	**> 250**	1.1 ± 0.37	1.79
	100 - 250	0.59 ± 0.12	
	< 100	0.1 ± 0.07	

The data represent the means and standard deviations of 3 replicated flasks over 3 passages.

3.4. EFFECT OF TWO BASAL MEDIA ON THE GROWTH OF CELL SUSPENSIONS DERIVED FROM TWO DIFFERENT CALLUS LINES OF CARROT CV. MOROT DUKE

The growth of suspension cultures was examined in two different basal media: Murashige and Skoog (MS) (1962) and modified Lin and Staba medium (LS) (1961) as described by Fujimura and Komamine (1979a).

Two individual callus lines (Morot Duke-8 and Morot Duke-39), isolated from carrot cv. Morot Duke, were studied in this experiment. Callus cultures of Morot Duke-39 had been grown on MS2 supplemented with 5 μM 2,4-D for 2 months, while those for Morot Duke-8 had been grown for the same period on MS2 supplemented with 0.5 μM 2,4-D. Suspension cultures derived from the callus lines were prepared as described in Chapter 2, Section 1.4.1 and subsequently 5 ml aliquots of the suspension cultures were inoculated into 45 ml of either MS2 or LS2 fresh medium containing 0.5 μM 2,4-D in 250 ml conical flasks. Three replicate conical flasks for each cell line were cultured. Growth of the suspension cultures was measured by determining CVS at 2-day intervals as described in Section 2.5. Results are displayed in Figure 3.3.

Analysis of variance shows that callus induced in MS2 supplemented with 5 μM 2,4-D (i.e. Morot Duke-39) produced suspension cultures which grew more slowly than callus induced in MS2 supplemented with 0.5 μM 2,4-D (i.e. Morot Duke-8) ($p < 0.01$). Also, the LS basal medium used for growth of the suspension cultures was significantly less effective than MS medium although the differences between the two media were relatively small ($p < 0.05$, see Appendix 10). Once again, MS medium supplemented with the lower concentration of 2,4-D for inducing the embryogenic cultures gave better results, as was found in Section 2.5. As a result of this experiment, MS basal medium

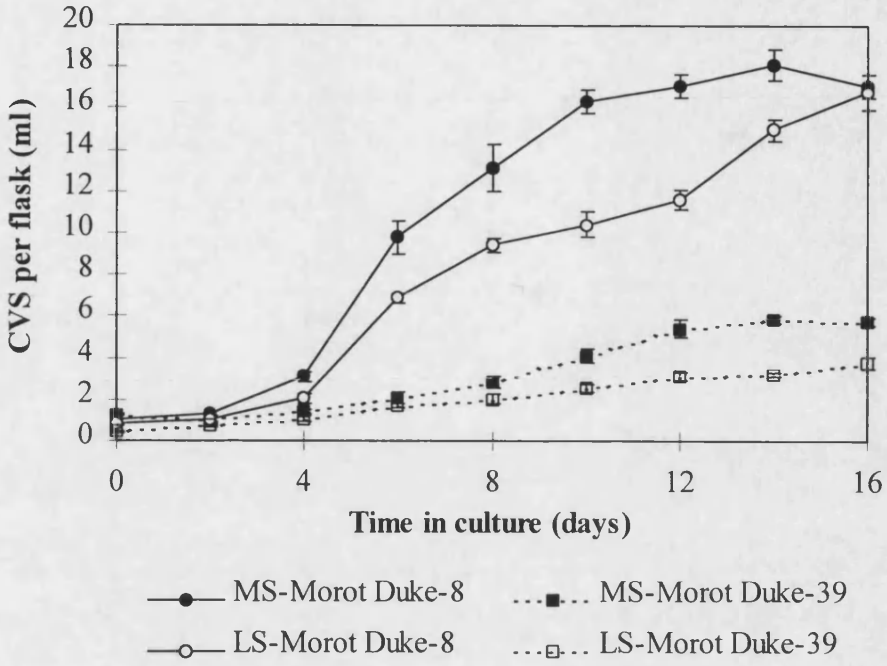


Figure 3.3: The effect of embryo induction and suspension culture maintenance media on subsequent growth of carrot cv. M. Duke suspension cultures.

Means of 3 replicated conical flasks are presented with standard deviation bars. Callus lines were initiated in either MS2 supplemented with 5 μM 2,4-D (Morot Duke-39) or MS2 supplemented with 0.5 μM 2,4-D (Morot Duke-8) for 2 months. Suspension cultures of two lines were maintained in both MS2 and modified LS2 medium containing 0.5 μM 2,4-D.

supplemented with 0.5 μM 2,4-D was chosen as the standard medium in the future experiments, for both the maintenance of suspension cultures and embryo induction.

3.5. REGENERATION OF MATURE SOMATIC EMBRYOS FROM SUSPENSION CULTURES

3.5.1. The Effects of Cell Unit Size and Regeneration Medium on Somatic Embryo Production from Long Term Suspension Cultures

In order to investigate the embryogenic competence of cell units from long term suspension cultures, tissue was removed from 7-month-old suspension cultures of carrot

cv. Morot Duke at the tenth day of the subculture cycle. The tissue was then split into four fractions by passing it through nylon sieves of 50 μm, 100 μm, 200 μm and 350 μm mesh size. No cells were retained by the 350 μm sieve. The density of the cell units in each fraction was then determined using a haemocytometer and adjusted to produce *ca.* 620 units per 0.5 ml. Aliquots of 0.5 ml from each fraction were then distributed onto the surface of the embryo development media on the basis of 10 drops per petri dish. The embryo development media tested were MS2, MS2 supplemented with 0.1 μM Z, MS2 supplemented with 0.5 μM 2,4-D and MS2 supplemented with 1% (w/v) AC. Three petri dishes for each treatment and each fraction size were set up. The number of mature somatic embryos (identified as green cotyledonary stage somatic embryos) produced from each 0.5 ml aliquot culture was counted after one month.

Table 3.8 shows that there were significant effects of fraction size and embryo development media on the production of mature somatic embryos from long-term suspension cultures ($p < 0.01$, see Appendix 11) and there were also significant interactions between fraction sizes and media. Although all the sizes of cell units were able to produce mature embryos on hormone free MS2 basal medium, the highest numbers were formed on MS2 supplemented with 0.1 μM Z. When the cell units were plated out onto MS2 medium with 0.5 μM 2,4-D or 1% AC, very few, if any, mature somatic embryos were formed (Plate 30, Chapter 3.III).

The highest numbers of mature embryos were regenerated from the 100-200 μm and 50-100 μm fractions with MS2 supplemented with 0.1 μM Z, although the results with these two fractions were not significantly different according to Duncan's Multiple Range Test at the 5% level. Overall, the lowest mature embryo formation was recorded from the < 50 μm cell units.

Table 3.8: The effects of embryo development media and the size of cell units on embryogenic competence of carrot cv. Morot Duke 7-month-old suspension cultures

Embryo Development Media	Mean number of mature embryo produced from 0.5 ml suspension			
	Fraction size (μm)			
	< 50	50 - 100	100 - 200	200 - 350
MS2 + 0.1 μM Z	8.4 a	30.9 a	31.4 a	22.2 a
MS2	4.0 b	24.2 b	31.9 a	11.9 b
MS2 + 1% AC	4.2 b	3.0 c	4.8 b	0 c
MS2 + 0.5 μM 2,4-D	3.7 b	0 c	0 b	0 c

Suspension cultures were maintained in MS2 supplemented with 0.5 μM 2,4-D and the number of mature somatic embryos was counted after one month in various embryo development media. Means within columns followed by the same letters are not significantly different according to Duncan's Multiple Range Test (p=0.05) and were calculated from 3 replicated samples.

3.5.2. The Effect of Pre-Treatment on Regeneration from Two-Month-Old Suspension cultures of Carrot cv. Morot Duke

The purpose of this experiment was to examine the regeneration capacity of two-month-old carrot suspension cultures, because of the possibility that the morphogenic competence of the suspension cultures might deteriorate with time before they could be used in the co-culture experiments.

Cell units below 100 μm in size were separated from two-month-old suspension cultures of carrot cv. Morot Duke maintained on MS2 containing 0.5 μM 2,4-D as described in Chapter 2, Section 1.4.1. 0.5 ml aliquots of the cell suspensions (the number of < 100 μm cell units *ca.* 760) were plated out, as explained previously, onto either MS2 or MS2 with 0.1 μM Z or MS2 with 0.5 μM 2,4-D medium. In addition, cell units were precultured for one week at a density of 1.0 ml PCV per 20 ml in 100 ml conical flasks containing the same range media before being plated out in a similar fashion (at a density of 590-660 cell units) onto the same media.

Four replicated petri dishes were established for each treatment and after four weeks the numbers of green cotyledonary somatic embryos were counted.

It can be seen from Figure 3.4 that there were no significant differences between the embryogenic competence of the cell units plated onto the three different regeneration media when no pre-treatment was used. The values of approximately 40 embryos per 0.5 ml were considerably higher than those achieved with older suspension cultures subjected to similar treatments (see Section 3.5.3). The highest values of embryogenic competence (up to 90 embryos per 0.5 ml) were achieved with cell units which had been pre-treated in either MS2 or MS2 with 0.1 μM Z liquid media before plating onto either of the same media. Cell units plated onto MS2 with 0.5 μM 2,4-D medium after any of the preculture treatments showed low embryogenic competence (< 10 embryos per 0.5 ml). As in the previous experiment, there was a small difference between the plating densities employed with the pre-treated and non-pre-treated cell units (*ca.* 590-660 cell units per 0.5 ml compared with *ca.* 760 cell units per 0.5 ml). It is unlikely however, that the enhanced embryogenic competence resulting from some of the pre-treatments could be attributed to these differences since, in this case, the pre-treated cell units received the lower plating densities.

3.5.3. The Effect of Pre-Treatments on Regeneration from Seven-Month-Old Suspension Cultures of Carrot cv. Morot Duke

The objectives of this experiment were to determine and improve the regeneration capacity of cell units less than 100 μm diameter from long term suspension cultures. It has often been reported that the frequency of somaclonal variants increases in long term callus and suspension cultures (e.g. Smith and Street, 1974; Giorgetti et al., 1995), including higher frequencies of disease-resistant variants (van den Bulk, 1991;

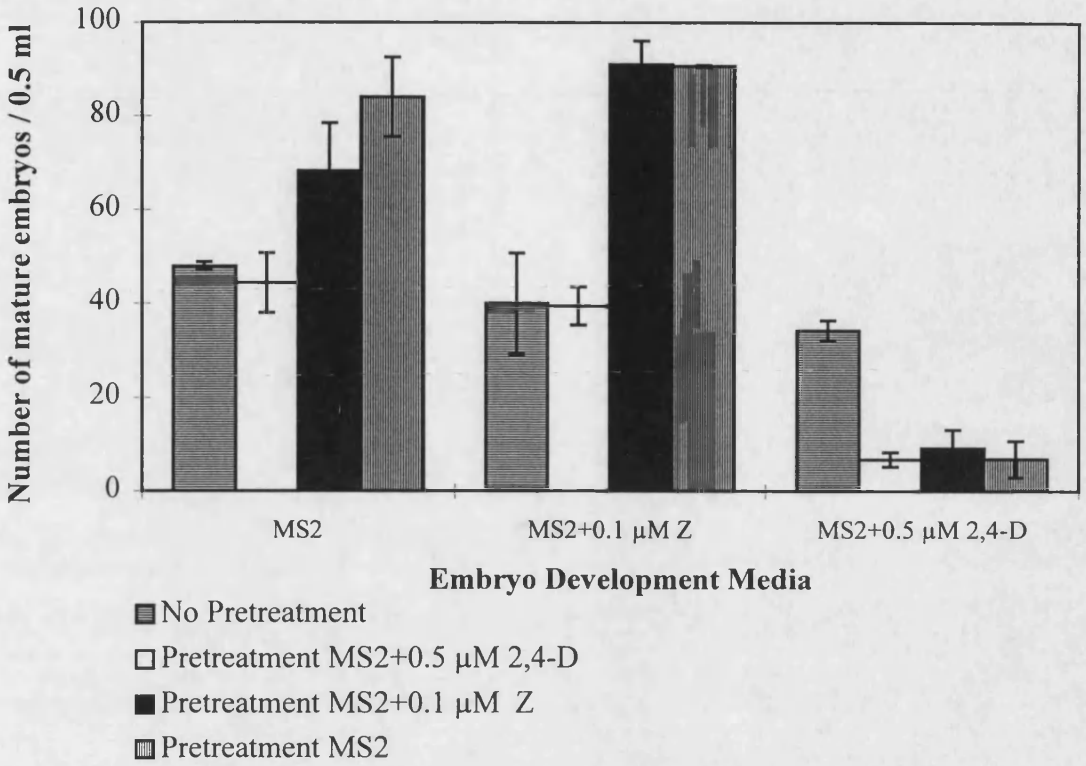


Figure 3.4: The effects of different pre-treatments on the embryogenic competence of < 100 µm cell units from two-month-old suspension cultures of carrot cv. Morot Duke

Means and standard deviations were calculated from five replicated petri dishes. Non-pre-treated suspension cultures maintained in 0.5 µM 2,4-D and suspension cultures pre-treated for one week in various liquid embryo development media were plated onto various semi-solid embryo development media. The number of mature somatic embryos produced from each 0.5 ml aliquot culture was counted after one month.

Krishnamurthi and Tlaskal, 1974) or of variants with enhanced levels of resistance (Larkin and Scowcroft, 1981).

Carrot cv. Morot Duke suspension cultures were maintained for seven months on MS2 containing 0.5 µM 2,4-D as described in Chapter 2, Section 1.4.1 and the same procedure for fractionation and plating was followed as with the two-month-old carrot suspension cultures in Section 3.5.2. A 0.5 ml aliquot of the suspension of cell units (the number of cell units *ca.* 510) was taken into a pipette and distributed onto the surface of

one of the four media tested (MS2, MS2 with 0.1 μM Z, MS2 with 0.1% AC and MS2 with 1% AC) on the basis of 10 equal drops per petri dish and this was repeated for each of the media. Alternatively, the < 100 μm cell units were subjected to a pre-treatment prior to plating onto the embryo induction media. The pre-treatment consisted of subculture of 1.0 ml PCV of the cell units in 100 ml conical flasks containing 20 ml of either MS2, MS2 with 0.1 μM Z, or MS2 with 0.5 μM 2,4-D medium for one or two weeks. At the end of the preculture period, 0.5 ml aliquots of the suspensions (*ca.* 620-880 units) were plated onto MS2, MS2 with 0.1 μM Z, MS2 with 0.1% AC or MS2 with 1% AC media as described above.

The test for embryogenic competence was based upon the mature somatic embryo count in cultures grown on the above media for 4 weeks. Five petri dishes were scored for the samples involving no pre-treatment and one week pre-treatment and 3 petri dishes for the two weeks pre-treatment.

Figure 3.5 shows the effects of the pre-treatments on the embryogenic competence of the cell units from the long-term suspension cultures. The embryogenic competence of cell units plated onto embryo induction media containing activated charcoal was generally very low (0 embryos per 0.5 ml) and the highest response (32 embryos per 0.5 ml) was obtained with cell units pre-treated for two weeks in MS2 medium before being subcultured onto MS2 supplemented with 0.1 μM Z medium. Generally speaking, the embryogenic competence of cell units pre-treated on the different media for either one or two weeks was, to varying degrees, higher than that of cell units which received no pre-treatment. It should be noted, however, that the inoculum densities employed with the pre-treated cell units were for technical reasons somewhat higher than those employed with the non-pre-treated cell units (*ca.* 620-880

units per 0.5 ml compared with *ca.* 510 units per 0.5 ml) and this could possibly have had a promoting effect on regeneration.

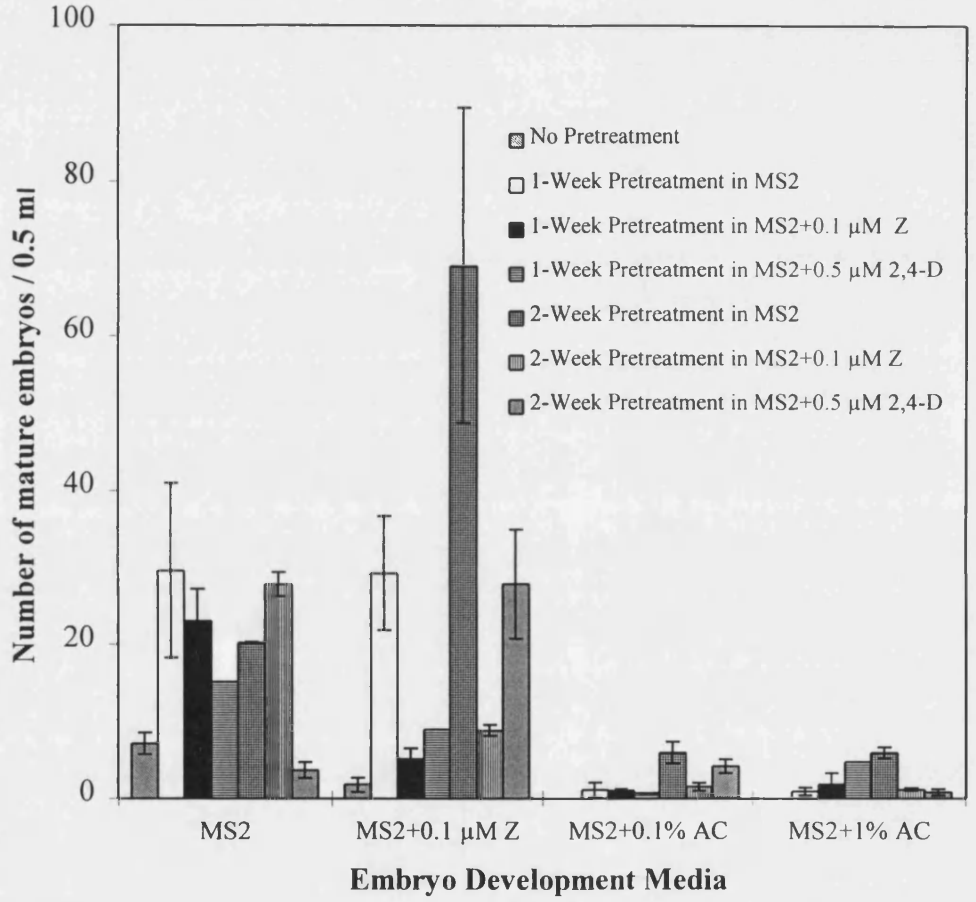


Figure 3.5: The effects of different pre-treatments on the embryogenic competence of < 100 µm cell units from long-term suspension cultures of carrot cv. Morot Duke

Non pre-treated suspension cultures maintained in 0.5 µM 2,4-D for seven months and suspension cultures pre-treated for either one week or two weeks in various liquid embryo development media were plated onto various semi-solid embryo development media. The number of mature somatic embryos produced from each 0.5 ml aliquot culture was counted after one month. Means and standard deviations were calculated from 5 replicated petri dishes for no pre-treatment and one week pre-treatment and 3 replicated petri dishes for two weeks pre-treatment.

Plate 5: Sequence in the development from carrot hypocotyl explant to carrot plants

(A) Callus initiation from hypocotyl explant of carrot cv. NRI-92 in solid MS2 + 0.5 μM 2,4-D after 1 week,

(B) highly embryogenic callus,

(C) growth of globular, heart-shaped and torpedo stage embryos on MS2 media,

(D) green cotyledonary stage embryos,

(E) plantlets grown in MS2 media on agar,

(F) plantlets grown in Fison MS2 compost,

(G) plants grown in the greenhouse,

(H) plants grown in the field,

(I) plants with flowers in the second cycle of biennial growth,

Plate 5(A)

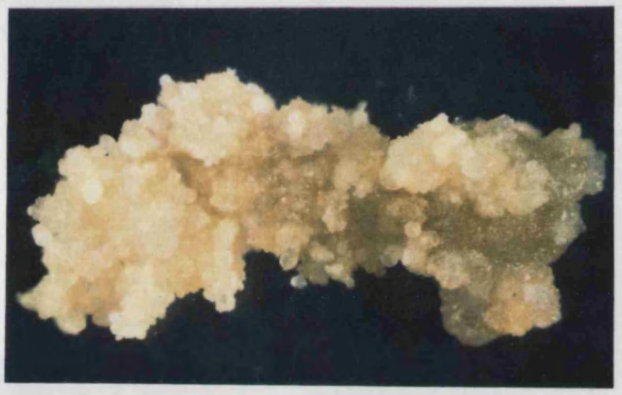


Plate 5(B)

Plate 5(C)

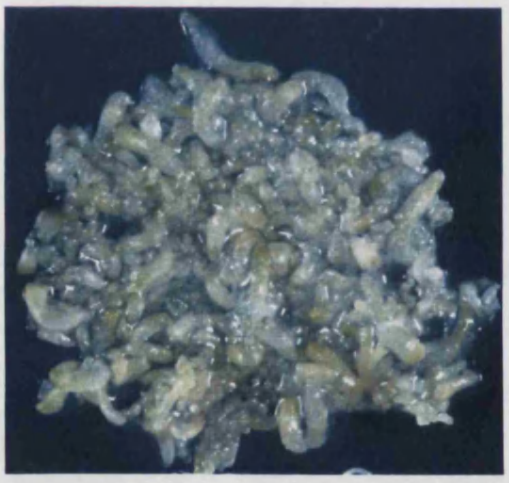


Plate 5(D)

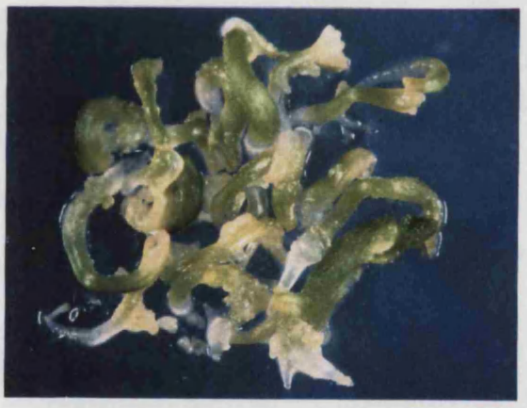


Plate 5(E)

Plate 5(F)

Plate 5(G)

Plate 5(H)

Plate 5(I)

3.6. ESTABLISHMENT OF PLANTLETS FROM MATURE SOMATIC EMBRYOS IN SOIL

Plantlet regeneration from mature somatic embryos and subsequent establishment in soil were proved to be highly efficient. Approximately 75-80% of the mature embryos being established produced plantlets when they were transferred to soil following the procedure described in Chapter 2, Section 1.5.

In summary, suitable carrot callus and suspension cultures were developed in this chapter in order to study *in vitro* selection for resistance to three major carrot diseases. Development of carrot plants from callus cultured cells is presented in Plate 5.

4. DISCUSSION

Optimised procedures for the production of somatic embryos and rapid production of regenerated plantlets from either callus or suspension cultures of carrot have been established in order to facilitate studies of the interaction between three major diseases of carrot and host tissue.

The production of embryogenic tissue was influenced by the age of seedlings from which the explants were taken and the type of explant. Cotyledon explants taken from younger seedlings (with hypocotyls only 1-3 mm) produced the highest numbers of somatic embryos per explant, compared with those taken from seedlings with 4-6 mm and 8-10 mm hypocotyls (Section 2.1.1).

Variation in regeneration capacity was found in explants taken from different regions of the cotyledons. Somatic embryo induction was higher (65%) from the basal region of the cotyledons in comparison with the terminal region (38%) (Section 2.1.1). Similar results were obtained by Cheng (1976, 1977) with cotyledon explants of Douglas fir (*Pseudotsuga menziesii* [Mirb.] Franco) plants which showed higher

regeneration (81%) in explants taken from the basal region compared with explants taken from the middle (69%) or terminal region (52%). The regeneration capacity was also higher in the proximal parts than in the distal parts of detached leaves of *Echeveria elegans* (Raju and Mann, 1971).

The wounding of cotyledon explants also had an obvious stimulatory effect on the somatic embryo production per explant. Yeoman and Forche (1980) suggested that wounding assists the rate of uptake and incorporation of added precursors from the medium, therefore it induces proliferation at the damaged surface of intact plants, but it is also possible that the "wound effect" itself could influence the embryogenic competence of cells in the immediate vicinity of the wound.

The average somatic embryo production was almost twice when 4-6 mm in length hypocotyl explants were cultured in embryo development medium compared to cotyledon explants taken from the same seedlings (Section 2.4). The embryogenic competence of hypocotyl explants taken from four of the cultivars tested was also extremely high (approximately twice) compared to cotyledons, although the two types of explant from cv. NRI-92 seedlings produced similar numbers of somatic embryos (Section 2.4 and Section 2.1). In conclusion, hypocotyl explants, 4-6 mm in length were generally used in this study to obtain embryogenic callus.

The seedlings from which the cotyledon and hypocotyl explants were taken and which were grown in the dark produced the highest amount of embryogenic callus, both in terms of the proportion of responsive explants and somatic embryo production. Light might have an important effect on the carbohydrate content of the explants as well as affecting the endogenous levels of auxins and inhibitory substances (Eliasson, 1971; Hansen and Eriksen, 1974).

The addition of 1% (w/v) activated charcoal to the embryo development medium had little effect on the production of somatic embryos compared to MS2 from both hypocotyl and cotyledon explants, but it was noticed that more torpedo and mature somatic embryos were produced on the medium containing activated charcoal. These results are in agreement with Warren and Fowler (1981) who reported that, in callus cultures of carrot, charcoal supplemented media did not support an increase in the number of globular embryos formed in the somatic embryos which were produced with high embryogenic competence but they initiated root formation from cultures of both low and high embryogenic competence. It was however observed in the later experiments (Section 3.5.3) that no mature embryos were formed from prolonged suspension cultures without pre-treatment in media containing activated charcoal. Similarly, Fridborg and Eriksson (1975) showed that, in ten-week-old suspension cultures of carrot, medium containing 1% (w/v) activated charcoal restored the capacity to regenerate plantlets while no plantlets formed in medium which lacked activated charcoal. Drew (1979) also reported similar results with cell suspensions of carrot.

Other studies have demonstrated that activated charcoal in the growth medium absorbed growth regulators added to the medium or inhibitory phenolic molecules secreted by the carrot tissue (Fridborg et al., 1978; Weatherhead et al., 1978). Consequently, it is possible that the amount of charcoal used in this study (Section 3.5.3 and 2.1) might have been insufficient to absorb all the inhibitory metabolites from the larger quantity of cells or medium. In this context, it is perhaps relevant that Drew (1979) reported that embryo formation with seven-month-old carrot suspension cultures in a medium containing 1% (w/v) activated charcoal was achieved when 1 ml of inoculum was employed but not when the inoculum size was increased to 6 ml.

The effects of MS and modified LS basal media were similar both on induction of somatic embryos from semi-solid medium and on the growth of suspension cultures (Section 2.2 and 3.4). In semi-solid form, both media produced callus containing single cells and numerous multicellular proembryogenic structures. When, however, the embryogenic callus taken from the modified LS medium was transferred onto auxin-free medium, well-formed globular, heart and a few torpedo-shaped somatic embryos were obtained in the present study (Section 2.2), but embryos failed to reach the plantlet stage. In contrast, when the same procedure was carried out with embryogenic callus produced on MS medium, somatic embryos formed plantlets. This effect of the modified LS medium, in comparison with the MS medium, might be due to the form of nitrogen in the medium, since MS medium contains 40 mM NO_3^- and 20 mM NH_4^+ while modified LS medium contains the same total amount of nitrogen but in the form of 55 mM NO_3^- and 5 mM NH_4^+ (Fujimura and Komamine, 1979a). It has been shown in many publications that the balance between nitrate and reduced nitrogen in the form of ammonium plays a critical role for initiation (Halperin and Wetherell, 1965; Halperin, 1966; Kamada and Harada, 1979) and maturation (Ammirato and Steward, 1971) of somatic embryos. Halperin and Wetherell (1965), for example, demonstrated that when petiole callus sieved through a 45-75 μm mesh was placed in 2,4-D free liquid culture, the formation of plantlets was evident two weeks after inoculation, but plantlet formation was very slow in medium lacking ammonium and varied from culture to culture. They concluded that the optimal nutritional circumstances for the formation of plantlets required further studies. The present findings suggest that MS medium appeared to be more effective than LS medium both in relation to the embryogenic response of callus cultures and to the maturation of somatic embryos.

Fourteen days incubation at the embryo induction stage on MS2 basal medium supplemented with 0.5 μM 2,4-D was chosen as the standard procedure for future experiments since, in comparison with the use of 5.0 μM 2,4-D, it aided somatic embryo production and improved the development of mature somatic embryos following transfer to auxin-free medium (Section 2.5). The reduced formation of green cotyledonary-stage embryos from hypocotyl explants, when using high concentrations of 2,4-D in the embryo induction medium, might have been a result of sensitivity to 2,4-D carry-over to the embryo development stage. Fujimura and Komamine (1979b), however, concluded that a critical level of exogenously supplied auxin might be necessary to increase the endogenous auxin level in cell units to the optimal level for induction of embryogenesis. In this context, the morphogenic responses of two callus lines of birdsfoot trefoil (*Lotus corniculatus* L.) to 2,4-D observed by Swanson and Tomes (1980) may be relevant since the morphogenically responsive callus line absorbed 2,4-D from the organogenic induction medium less strongly than the less responsive line and released 2,4-D more rapidly on transfer to differentiation medium. This could mean that the less responsive line accumulates too much 2,4-D to allow organogenic induction in the first medium and then retains 2,4-D at a level which inhibits differentiation capacity following transfer to the second medium. Montague et al. (1981a; b) working on the metabolism of 2,4-D in highly embryogenic carrot cells and in non-regenerable soybean cells found that the soybean cells conjugated a large amount of absorbed 2,4-D to amino acids whereas the carrot cells contained free 2,4-D. As a result, the soybean cells did not release 2,4-D when transferred to the auxin-free medium and it was concluded that the retention of 2,4-D resulted in the loss of morphogenic competence.

Newcomb and Wetherell (1970) reported that embryogenesis in wild carrot was improved first by washing the cells with MS medium to remove the majority of 2,4-D prior to transfer to an embryo development medium containing 2,4,6-T (the anti-auxin, 2,4,6-trichlorophenoxyacetic acid). In contrast, Fujimura and Komamine (1979b) found that when carrot cells (47-81 μm sizes) were cultured in liquid LS medium contained either 2,4-D (0.5 μM) or 2,4,6-T (10 μM) for embryo development, somatic embryo formation was highly inhibited compared to LS basal medium free from growth regulator. They concluded that the high amount of auxin could be loaded to the plant cells and therefore anti-auxin supplemented media may not eliminate the inhibitory effect of residual exogenously supplied auxins. The effects of the various growth regulators on the embryogenesis of carrot cells were examined by Fujimura and Komamine (1975) who reported that 2,4-D (> 10^{-9}M), IAA (>10^{-10}M) and cytokinins, such as BAP and KIN added into modified MS liquid medium for embryo development inhibited embryo formation of carrot cells (47-81 μm sizes) while Z promoted the embryogenesis at a concentration of 0.1 μM. In addition, Gibberellin A_3 and ABA blocked the embryogenic development at the heart shape stage.

In the present study, four different cultivars were compared for their somatic embryo production on semi-solid medium and found to vary in their embryogenic competence. Only Morot Duke and Morot Favor gave relatively high values with Morot Duke having the highest rate of somatic embryo production after incubation for one week in embryo induction medium. However, the cultivar, Morot Rondino produced the highest number of mature embryos in the embryo development medium, following induction with 0.5 μM 2,4-D. This contrasts with the very low rate of growth in suspension culture of this cultivar (see Section 2.4 and 2.5). Tianran and Neumann (1985), have also reported that embryogenic competence varied among eight carrot

cultivars (Wild carrot, Egyptian Black, Indian Red, Indian Black, Vosgeses, Rotin, Lobbericher, Rote Riesen) that were investigated. Schäfer et al. (1985) also confirmed the influence of genotype on embryogenic competence in carrot since petiole explants derived from cultivar Rotin were highly embryogenic while those from the cultivars Lobbericher failed to produce embryos. In this case the observed variation in embryogenic competence indicated differences in the genetic systems of two varieties which were reflected as observed differences in their endogenous hormonal systems. Krikorian (1982) also found differences in the embryogenic competence of tissues of carrot cultivars grown in suspension cultures and he pointed out that different samples of wild carrot *Daucus carota* var. *carota* (Queen Anne's Lace) were more consistent in their responsiveness than different cultivars of cultivated carrot. It is therefore possible that the difference between four cultivars used in this study could be due to the manner in which they metabolise the 2,4-D supplied in the medium and how this effects their endogenous hormonal systems. These differences are then reflected in the levels of embryogenic tissue production at embryo regeneration.

A typical sigmoid growth curve with a lag phase followed in turn by acceleration, exponential, deceleration and stationary growth phases are characteristic of cell suspension cultures (Street, 1973). The two sigmoid growth curves from cvs. Morot Rondino and Morot Favor, obtained by measurement of CVS in the present study, were in agreement with the results of Krogstrup (1990) who also monitored Sitka spruce (*Picea sitchensis*) suspension cultures by measuring settled cell volume. Similar results were also obtained by Blom et al. (1992) with 10 different cell lines from a range of species including *Cinchona robusta, Catharanthus roseus*, *Humulus lupulus* and *Digitalis lanata.* A linear correlation between the settled cell volume, packed cell volume, fresh weight was also reported for pear suspension cultures (*Pyrus communis*

L.) by Ryu et al. (1990). Although Morot Duke and Morot Ingot did not reach the stationary phase in 14 days culture by measurement of CVS, the viability of their suspensions declined very rapidly 12 days after inoculation. This may reflect the fact that Morot Duke and Morot Ingot have indeed reached their stationary phase by day 12-14 and possibly that the continual increase in CVS after this time is due to the increase in cell size through vacuolation rather than multiplication. Further observations, however would have to be carried out to confirm this hypothesis. It was therefore concluded that the proliferation of carrot suspension cultures could be routinely and non-destructively quantified by use of the CVS in carrot suspension cultures, although it was recognised that this did not necessarily reflect accurately the changes in cell number.

In order to use cell suspensions for co-culture experiments with pathogenic organisms it is important to have information about the distribution of cell unit sizes with the cultivars and the morphogenic competence of the different sizes of cell units. An experiment (Section 3.1) with the four cultivars Morot Duke, Morot Favor, Morot Ingot and Morot Rondino showed that the highest growth rate was obtained with cv. Morot Duke, followed by cv. Morot Ingot; in all of the cultivars after three 16-day passages and the fraction containing the smallest cell units (< 50 μm) were relatively small. Ideally, co-culture experiments should be carried out with the smallest size cell units possible that are morphogenically competent, so that a relatively large proportion of the cells within the units are able to come into contact with the pathogen or with its product(s). In another experiment (Section 3.5.1), therefore, the embryogenic competence of the different fractions of cell units was measured and it was shown that the smallest cell units (< 50 μm) produced the fewest mature embryos when transferred to the semi-solid MS2 medium. It is possible that the smaller cell units require further

developmental stimuli to continue their growth and development. Steward et al. (1975) showed that < 150 μm cell units from carrot suspension cultures, which did not grow in the light over a period of four weeks, grew well in the dark in MS liquid medium which had been pre-conditioned by the prior growth of an abundant crop of somatic embryos. In this study (Section 3.5.1) it was shown that the larger cell units (50-100 μm and 100-200 μm) were more morphogenic than the 50 μm units, with the 50-100 μm fraction being particularly active on the basis of the number of mature embryos produced per unit volume of tissue. It was decided therefore to adopt a standard fraction size of < 100 μm for use in the co-culture experiments.

It appears that the regeneration capacity of suspension cultures declined with the serial subculture since seven-month-old cultures, without any pre-treatment, produced very few plantlets in comparison with the two-month-old cultures (Section 3.5.3). These results were in agreement with those of Smith and Street (1974) who showed that both callus and suspension cultures of carrot declined in their embryogenic competence following continued serial subculture. Plant cells were found to become more sensitive to inhibition of their capacity to form somatic embryos by 2,4-D and that the loss of embryogenic potential was found to be correlated with the development of polyploid and aneuploid cells in the culture. Sussex and Frei (1968), however, showed that long term (several years) callus cultures of carrot grown on a medium containing coconut milk retained the ability to produce somatic embryos when transferred to simpler liquid culture medium lacking coconut milk but not when transferred to agar medium of the same composition. In addition, Meyer-Teuter and Reinert (1973) demonstrated that slow growing long-term carrot callus cultures retained their ability to form somatic embryos longer than fast growing cultures and Warren and Fowler (1981) suggested that long-term carrot suspension cultures may lose their embryogenic competence as a result

of the release and accumulation of inhibitor(s) which suppress the formation of somatic embryos.

In the present study, either one week or two weeks pre-treatment of long-term suspension cultures in liquid MS2 or MS2 supplemented with 0.1 μM Z prior to transfer to semi-solid embryo development medium of the same compositions increased the embryogenic competence of seven-month-old suspension cultures by 35 times (two-week preculture in MS2 medium followed by transferring onto MS2 supplemented with 0.1 μM Z). Since these pre-treatments employed liquid media of the same compositions as those used for the semi-solid embryo development media, it would seem that the beneficial effects of the pre-treatments were more likely to be a consequence of the physical conditions with the liquid media than of the nutrient conditions. There are various possible explanations for such an effect, one of which could be more rapid unloading of the 2,4-D which is likely to have accumulated in the cells during their long term maintenance as suspension cultures. The effects of pre-treatment and auxins for regeneration of somatic embryos from single cells were reported by Nomura and Komamine (1985). They showed that one week preculture of single cells in auxin-free media resulted in no embryo formation in embryo-inducing medium, but preculture of single cells in medium containing either 2,4-D or Z at 0.05 μM induced formation of embryogenic cell clusters. Fujimura and Komamine (1975; 1980a) also showed that somatic embryogenesis of carrot can be strongly promoted by Z in the first 3-4 days of culture during which active cell division occurs.

The appearance of regenerated mature somatic embryos at a high number from newly established suspension cultures (two-month-old) without any pre-treatment was observed and this result suggests that the freshly isolated cells are less sensitive to inhibition of embryogenesis by auxin (Smith and Street, 1974).

CHAPTER 3

RESULTS AND DISCUSSION

II - PATHOGENICITY OF *ERWINIA* SPECIES, *P. VIOLAE* AND *E. HERACLEI* ON SUSCEPTIBLE CARROT VARIETIES

1. INTRODUCTION

Some pathogens reproduce on living hosts (biotrophic pathogens) while others cause rapid cell damage and invade moribund tissues. The pathogens used in this work, *Erwinia* spp., *P. violae* and *E. heraclei* were chosen because of their economic significance but also because they represent a continuum from the extreme necrotrophic strategy of soft rot erwinias, to the haustorium-forming obligately biotrophic powdery mildews; the mode of parasitism of *P. violae* is not known but appears to lie somewhere between.

The purpose of this chapter was to confirm and compare pathogenicity of isolates and to optimise the inoculation conditions for later assessment of the susceptibilities of different somaclones derived from tissue culture. Mechanisms of pathogenicity or infection of each pathogen was also studied. This was in order to provide possible pathogenicity factors for use as agents in *in vitro* screenings for resistance as well as to

provide likely targets for resistance mechanisms of any resistant somaclones that may have resulted from this work.

2. *Erwinia carotovora* subspp. AND *E. chrysanthemi*

2.1. DEVELOPMENT OF INOCULATION METHODS

Ecc (SCRI 1039) and *Eca* (SCRI 139) suspensions were grown in LB broth overnight and 10 fold dilution series (10^9 to 10^6 cfu/ml) were made with sterile distilled water. A cork borer (0.5 cm diam.) was used to make 5 mm and 15 mm deep wounds at five different sites along carrot taproots. 20 µl bacterial suspension containing 10^9, 10^8, 10^7 and 10^6 cfu/ml and sterile distilled water as a control were inserted randomly in disposable pipette tips (200 µl) in randomised design. Inoculated carrots were sealed with Vaseline. Four moist chambers with 5 carrot taproots for each treatment were incubated in the dark at 22 ± 1 °C and 98% RH in a growth chamber. Diameter of lesions was measured 2 and 4 days after inoculation.

It is apparent from Table 3.9 and analysis of variance that infection was significantly influenced by *E. carotovora* strains, depths and concentrations when roots were incubated for 2 and 4 days ($p < 0.01$, Appendix 12a, 12b).

Pathogenicity was greater with increasing inoculation concentrations. Neither *Ecc* nor *Eca* produced any symptoms at 10^6 cfu/ml while 10^9 cfu/ml produced substantial rotting. Bacteria started to produce water soaked lesions on taproots within 2 days and by 4 days lesions often girdled the entire taproot.

Deep 15 mm wounds resulted in much more severe rotting than the 5 mm wounds. The *Ecc* strain was more pathogenic than *Eca* (Plate 6). The minimum concentration of *Ecc* which gave reproducible infection and therefore could allow

detection of resistant somaclones, was 10^7 cfu/ml *via* 15 mm wounds, hence these conditions were employed in subsequent experiments.

Table 3.9: Optimisation of inoculation of carrot taproots with *E. carotovora*

Strains	Wounding depth (mm)	Inoculum concentrations	Mean diameter of rotted area (cm)
Ecc	5	10^6	0
		10^7	0.42 ± 0.21
		10^8	2.54 ± 0.50
		10^9	4.04 ± 0.40
	15	10^6	nd
		10^7	2.37 ± 0.30
		10^8	4.19 ± 0.67
		10^9	4.89 ± 0.33
Eca	5	10^6	nd
		10^7	0
		10^8	0.38 ± 0.62
		10^9	2.71 ± 1.02
	15	10^6	nd
		10^7	0.78 ± 0.21
		10^8	1.96 ± 1.31
		10^9	4.18 ± 0.68

Ecc: *E. carotovora* subsp. *carotovora* ***Eca:*** *E. carotovora* subsp. *atroseptica*
nd: not determined. Data are the mean and standard deviation of four replications of five carrot roots 4 days after inoculation.

2.2. PATHOGENICITY OF *E. carotovora* AND *E. chrysanthemi* WILD TYPE AND MUTANT STRAINS

In order to investigate pathogenicity and to understand the mechanisms of cell killing (Chapter 3.III), *Erwinia* mutants with impaired production of extracellular enzymes were obtained and tested. *E. carotovora* subsp. *carotovora (Ecc)* and the related soft rot pathogens *E. carotovora* subsp. *atroseptica (Eca)* and *E. chrysanthemi (Ech)* wild type and mutants were supplied from 4 different research centres. These were Scottish Crop Research Institute (SCRI) (McMillan et al., 1994), Universities of Warwick (Mulholland et al., 1993; Reeves et al., 1993), Missouri (Murata et al., 1990) and Cornell (Collmer et al., 1985).

Carrots from a local source were prepared and placed 5 per box in two moist chambers in the first experiment and four in the second. They were then inoculated randomly with bacteria at 10^9 or 10^7 cfu/ml in wounds made in upper, mid or lower sections of tap roots. Lesion diameters were determined after 5 days at 22 ± 1 °C.

2.2.1. *Ecc* and *Eca* (ex. Warwick University)

The mutants of *Ecc* and *Eca* used in the experiment were RJP 116, RJP 243, PFP 16, PR 54, ATTn5, GS 7000, MS 10 and 94.15. Also three wild types SCRI 1043, SCRI 1039, SCRI 193 were compared for pathogenicity.

Pathogenicity to carrot roots of mutants and parent wild type are shown in Table 3.10 and Plate 7 which demonstrate that whilst the wild type strains (*Ecc*) produce the characteristic tissue rotting at 10^9 and 10^7 cfu/ml, no rotting or less rotting occurred with mutants impaired in their ability to produce extracellular enzymes.

When the non-pathogenic bacterial strains were omitted from the statistical analysis, there were considerable differences between strains and concentrations ($p < 0.01$, Appendix 13a). It can be noticed that the pectinase over-producing mutant PFP 16 caused greater rotting than wild type SCRI 193 at 10^9 cfu/ml. Neither wild type nor mutant of *Eca* produced rotting at the concentrations used.

It is apparent that extracellular enzyme production can have marked influence on pathogenicity.

2.2.2. *Ecc* (ex. Missouri University)

Four mutants of *Ecc* called AC 5010, AC 5013, AC 5017, AC 5031 and wild type strains *Ecc* 71 and SCRI 193 were studied. Carrot roots were prepared as explained in Chapter 2 (Materials and Methods), Section 3.

Table 3.10: Determination of virulence of different mutants and wild types of *Ecc* and *Eca* at 10^9 and 10^7 cfu/ml inoculum concentrations

		Lesion diameter (cm)	
Strains	**Nature of mutation**	**10^7 (cfu/ml)**	**10^9 (cfu/ml)**
SCRI 1043	**Wild Type**	0	0
94.15	**Tn5-induced-reduced virulence mutant *mopE*$^-$**	0	0
MS 10	**Lac$^-$ *carI*$^-$ from ATTn5**	0	0
RJP 243	**rex$^-$ (Non-rex*I*) (Pel Cel Prt ↓)**	0	0
RJP 116	**rex*I*$^-$ (Pel Cel Prt ↓)**	0 a	1.42 a
PR 54	**Tn*phoA*-induced Out$^-$**	0 a	2.17 a
ATTn5	**Carbapenem producer Tn-5 induced RM mutant**	1.71 ab	2.59 a
GS 7000	**EMS-induced Cel$^-$**	1.67 ab	2.73 a
SCRI 193	**Wild Type**	2.47 bc	4.53 b
PFP 16	**Tn*phoA*-induced hyper (Pel Cel Prt ↑)**	2.95 bc	5.16 bc
SCRI 1039	**Wild Type**	4.26 c	6.27 c

Out$^-$: non secretory mutants **rex*I*$^-$**: down regulated exoenzyme inducer mutant **Pel:**Pectate lyase **Cel:** Cellulase **Prt:** Protease **non-rex*I*$^-$**: non regulatory mutant. Means within columns followed by the same letters are not significantly different according to Duncan's Multiple Range Test (P= 0.05). Each value is the mean of two replications of ten carrots.

Table 3.11: Determination of virulence of different mutants and wild types of *Ecc* at 10^7 and 10^9 cfu/ml inoculum concentrations

		Lesion diameter (cm)	
Strains	**Nature of mutation [a]**	**10^7 (cfu/ml)**	**10^9 (cfu/ml)**
SCRI 1039	**Wild Type**	3.90 a	5.82 a
***ECC* 71**	**Wild Type**	0.03 b	0.15 b
AC 5031	**AepA::lacZ, Km^r**	0	0
AC 5017	**out::Tn10::lacZ(Tc^r)**	0	0
AC 5010	**out::Tn 5(Km^r)**	0	0
AC 5013	**out::Tn10(Tc^r)**	0	0

[a] : Abbreviations; **Km^r**: kanamycin resistant; **Tc^r** : tetracycline resistant. Means within columns followed by the same letters are not significantly different according to Duncan's Multiple Range Test (P= 0.05). Each value is the mean of four replications of five carrots.

Plate 6: **Carrot roots inoculated with *Ecc* and *Eca* bacterial strains. Note: *Ecc* produced more extensive rotting from 15 mm wounds than *Eca***

Plate 7: **Carrot roots inoculated with wild type and mutant bacterial strains of *Ecc*. Note: typical rot symptoms caused by wild type (SCRI 193), reduced symptoms with 3 mutants under producing pectate lyase (e.g. RJP 116, RJP 243) and enhanced symptoms with over producing mutant PFP 16, 7 days after inoculation**

ECA *ECC*

Plate 6

SCRI 193 | GS 7000 | PR 54 | RJP 116 | RJP 243 | PFP 16 | ATTn5 | MS10 | SCRI 1039

Plate 7

The results given in Table 3.11 show that, in contrast to extensive rotting caused by wild type SCRI 1039, the parent wild type *Ecc* 71 was weakly pathogenic and all derived mutants were non-pathogenic.

The *Ecc* strains had a significant ($p < 0.01$) effect on the disease symptom development as did the inoculum concentrations and the interactions between strain and concentrations ($p < 0.01$, Appendix 13b). Bacterial strains which did not produce rotting on carrot roots were not included in the analysis of variance.

Since mutants and wild types were not pathogenic to carrot roots they were omitted in subsequent experiments.

2.2.3. *E. chrysanthemi* (ex. Cornell University)

The mutants used above are probably pleiotropic in that several enzyme systems may be affected by mutations influencing secretion systems. Site directed mutagenesis with *E. chrysanthemi* has resulted in mutants lacking one or several pel genes (Collmer et al., 1982; 1985). These should give less equivocal results and were therefore tested for pathogenicity.

Four *Ech* mutants (CUCPB 5019, CUCPB 5018, UM 1004 and UM 1005), one *Ech* wild type (AC 4150) and one *Ecc* wild type (SCRI 1039) were used in this experiment. Carrot roots were prepared as explained in Chapter 2, Section 3.

Inoculated areas showed water-soaked lesions which appeared after 2 days. *Ecc* wild type was much more pathogenic than *Ech* wild type which resulted in few or no symptoms (Table 3.12). All pel mutants were less pathogenic than wild type. Greatest reduction in pathogenicity resulted from simultaneous loss of pel A, B and C.

Rotting varied significantly ($p < 0.01$) between the different *Erwinia* strains. Inoculum concentrations also affected symptom development significantly ($p < 0.01$, Appendix 13c).

Since mutants and wild types of *Ech* were far less aggressive than *Ecc* strains to carrot roots they were not used in subsequent experiments.

Table 3.12: Determination of virulence of different mutants and wild types of *Ech* at 10^7 and 10^9 cfu/ml inoculum concentrations

Strains	Nature of mutation [a]	Lesion diameter (cm)	
		10^7 (cfu/ml)	10^9 (cfu/ml)
CUCPB 5019	**PelABC⁻ Km^r**	0.07 a	0.37 a
UM 1004	**PelAE⁻ Km^r**	0.16 ab	0.26 a
CUCPB 5018	**PelBCE⁻ Km^r**	0.45 ab	0.75 ab
UM 1005	**PelABCE⁻ Km^r**	0.60 ab	0.72 ab
AC 4150	**Wild Type**	0.97 b	1.60 b
SCRI 1039	**Wild Type**	3.67 c	4.70 c

[a] : Abbreviations; **Km^r** : kanamycin resistant **Pel** : pectate lyase. Means within columns followed by the same letters are not significantly different according to Duncan's Multiple Range Test (P= 0.05). Each value is the mean of five replications of five carrot roots.

2.3. GROWTH OF *Ecc* WILD TYPE AND MUTANTS IN CARROT TAPROOTS

The aim of this experiment was to examine the relationship between number of bacteria and pathogenicity to carrot roots.

Carrot roots were obtained locally. The *Ecc* isolates used were SCRI 193, PFP 16, RJP 116, RJP 243 (ex. Warwick University). The procedure for carrot roots and bacterial inoculum preparation were as explained earlier.

Carrot roots were inoculated with 10^7 and 10^9 cfu/ml bacteria as explained in Chapter 2, Section 3. Four moist chambers with 5 carrot taproots were incubated in the dark at 22 ± 1 °C and 98% RH in a growth chamber.

The number of bacteria in macerated roots were counted 4 days after inoculation. Ten randomly chosen rotted areas infected with 10^7 cfu/ml were excised and comminuted in a sterile petri dish by a glass pestle. Samples were then removed into 30 ml sterile universals and vortex mixed for 5 to 10 minutes. One ml of this homogenated sample was added to 9 ml LB broth. Samples were diluted to give 10^6 and 10^7 cfu/ml. The number of viable bacteria was estimated by spread plating 100 µl of 3 samples into 3 petri dishes containing NA and incubated for 2 days at 30 °C. The number of colonies formed was then counted.

Table 3.13: Growth of *E. carotovora* wild type and mutants in carrot roots

Strains	Time (days)	
	0	4
	Bacterial growth ($\times 10^7$ cfu/ml)	Bacterial growth ($\times 10^9$ cfu/ml)
RJP 116	2.00 a	2.80 a
RJP 243	2.63 a	4.04 ab
SCRI 193	2.40 a	8.86 c
PFP 16	9.00 b	4.43 b

Means within columns followed by the same letters are not significantly different according to Duncan's Multiple Range Test (P=0.05). Means were calculated from 3 replicated samples.

It can be observed from Table 3.13 that the number of bacteria of all *Ecc* strains in the carrot roots increased over 4 days with time irrespective of their pathogenicity. The highest multiplication rate occurred with SCRI 193 (350 Fold). The populations of RJP 116 and RJP 243 increased approximately 140 fold. However, population growth of PFP 16 increased 50 fold over the same period. The number of bacteria were significantly different between the bacterial strains ($p < 0.01$, Appendix 14). Previous results (Section 2.2.1) demonstrated that PGL over-producing mutant, PFP 16, was more pathogenic in comparison with wild type SCRI 193. Yet it was present in lesions

in significantly lower numbers. This may indicate that aggressiveness of PFP 16 may be partly dependent on the greater production and secretion of extracellular enzymes rather than dependent on increased growth.

2.4. DETERMINATION OF PECTATE LYASE PRODUCTION FROM *E. carotovora in vivo*

Pectic enzymes which have frequently been shown to be involved in pathogenicity of necrotrophic pathogens (Cooper, 1984) are released in large quantities by *Erwinia carotovora* spp. One of the predominant pectic enzymes secreted by soft rot *Erwinia* spp. is PGL (Kotoujansky, 1987; Ried and Collmer, 1986; Salmond, 1994). Enzyme deficient mutants are of clear value in studies on mechanisms of pathogenicity. Many mutations that affect synthesis or secretion of extracellular enzymes, including pectinases, cellulases and proteases lead to a reduction or loss of virulence *in planta* (Murata et al., 1990). In view of this, the presence of PGL from *Ecc* wild type and mutants in carrot taproots and its effect on embryogenic suspension cultured cells were examined.

2.4.1. Extraction of PGL from *Ecc* Inoculated Carrot Taproots *in vivo*

The purpose of this experiment was to detect the possible production of bacterial PGL during infection of carrot roots.

Carrot roots were prepared and inoculated as described in Chapter 2, Section 3. Bacteria used in this experiment were SCRI 193 (wild type), RJP 116 (PGL deficient mutant), RJP 243 (PGL deficient mutant) and PFP 16 (PGL over producing mutant). 10^7, 10^9 cfu/ml bacteria and sterile distilled water as a control were inoculated into taproots. The lesions that developed around the inoculated area were removed after 4 days and extracted for enzyme. Controls consisted of uninfected tissue. Extractions were

done as described in Materials and Methods, Section 8. Pectate lyase activity was assayed (Materials and Methods, Section 6.1) spectrophotometrically at 235 nm in extracted samples (Dow et al., 1987).

Table 3.14 demonstrates that all strains of *Ecc* produced PGL in carrot roots. However, the over-producing mutant (PFP 16) produced twice as much PGL as wild type (SCRI 193). Less PGL occurred with enzyme deficient mutants (RJP 116, RJP 243) but activities were still significant (*ca.* 50% of that produced by wild type).

Table 3.14: PGL production by *Ecc* wild type and mutants

Strains	PGL production* (μkat)
SCRI 193 (wild type)	2.46 ± 0.31
PFP 16 (PGL ↑)	4.35 ± 0.62
RJP 116 (PGL ↓)	0.97 ± 0.09
RJP 243 (PGL ↓)	1.09 ± 0.17
Uninfected tissue	0.02 ± 0.005

* Pectate lyase (PGL) activity assayed by UV absorbance at 235 nm. Extractions obtained from 4-day-old macerated tissue. Enzyme assays were repeated three times; the values shown with standard deviations are from a mean of 3 representative assays.

2.5. SCREENING SOMACLONES FOR DISEASE RESISTANCE TO *Erwinia carotovora*

The purpose of the experiment was to test disease resistance or tolerance to *E. carotovora* of regenerated taproots from 2-month-old embryogenic suspension cultures of carrot.

Hypocotyl explants taken from hybrid F1 carrot cv. Morot Duke were placed on the surface of MS2 medium containing 0.5 μM 2,4-D to induce callus formation. Embryogenic callus was proliferated and maintained by subculturing into embryo induction media (MS2 + 0.5 μM 2,4-D). Carrot suspension cultures were initiated as in Chapter 2, Section 1.4.1. Two-month-old suspension cultures were homogenised by

sieving and < 100 μm cells were collected. Mature somatic embryo formation was initiated by placing 0.5 ml (the number of cell units *ca.* 760) of embryogenic carrot suspension cultures in 10 drops onto the surface of MS2 agar medium. Cultures were incubated at 25 ± 1 °C under light with a 16 hour photoperiod. When carrot plantlets reached sufficient size they were transplanted to soil (10-12 cm in length) and were acclimatised for two months; plantlets were then transferred to the field (Bath University Field Station) to produce taproots. Regenerated carrot tap roots were harvested 4 months after planting in the field and were inoculated on the same day. *Ecc* SCRI 193 was prepared as explained before. Two hundred and twenty individual carrot roots were prepared as described in Materials and Methods, Section 4.2 and were inoculated with 20 μl containing 10^7 cfu/ml at a depth of 15 mm. They were then placed into moist chambers (10 roots per moist chamber) and incubated at 98% RH, 22 ± 1 °C in the dark for a week. The diameter of rotted area, the percentage of uninfected roots and percentage of inoculated sites that gave lesions were determined.

Table 3.15: Pathogenicity of *E. carotovora* on regenerated taproots of carrot cv. Morot Duke

Regenerated tap roots	% taproots uninfected	% inoculated sites infected	Mean diameter of rotted area (mm)
Morot Duke (Original seed line)	18.2	68.2	6.0 ± 5.80
Taproots regenerated from Morot Duke	3.2	87.1	12.5 ± 7.50

Data are the mean and standard deviations of 220 individual regenerated roots.

Table 3.15 shows that taproots regenerated from Morot Duke were much more susceptible compared to the original seed line. The severity of infection by *Ecc* was higher on the regenerated roots than tap roots from original seed line. Also, 3.2% of the

inoculated individual roots of regenerants remained symptomless compared with *ca.* 18% of the original seed line.

3. INOCULATION METHODS WITH CARROT ROOTS AND *P. violae*

3.1. THE EFFECT OF TAPROOT SIZE AND INOCULATION SITE ON CAVITY SPOT FORMATION

The purpose of the experiment was to develop a method to obtain reproducible cavity spot lesions and to optimise the inoculation conditions for the assessment of the susceptibilities of different somaclones derived from tissue culture.

Tap roots of carrot cv. Burton (Nickerson Ltd.) were collected from Mortimer's, farm, Bromham, Wilts and inoculated on the same day as they were harvested.

P. violae was grown at room temperature (17-21 °C) for 10 days on CMA agar. The roots were divided into three groups according to size; small (8-10 cm length), medium (11-15 cm length) and large (16-20 cm length). Five carrot roots per moist chamber and seven replicate chambers for small and medium sizes and three replicate chamber for large size carrot roots were set up and then incubated for 10 days at 90% RH in the dark at 20 ± 3 °C. Non-inoculated agar plugs were placed on roots as the control. Five days after inoculation the plugs were removed. Cavity spots were assessed by measuring the diameter of lesions and percentage of sites that resulted in infection after 10 days incubation.

Water soaked lesions, 2 days after inoculation, were first evident on the surface of the taproots, then these lesions became sunken and elliptical but did not spread into inner layers on the roots (see Plate 8 and Plate 9).

The percentage mean of sites infected was 59-68% for all sizes (see Table 3.16). There was no significant difference in frequency and extent of cavity spot formation

Plate 8: Water soaked lesions 2 days after inoculation with *P. violae*

Plate 9: Detail of cavity spot lesion 2 days after inoculation with *P. violae*

Plate 8

Plate 9

between three sites while inoculum sites had a considerable effect on the cavity spot production per root ($p < 0.01$, Chi-Square Test Appendix 15a, 15b). The upper region of carrot taproots had significantly more cavities than the middle and bottom regions.

Table 3.16: Development of cavity spot at different locations on carrot roots of different sizes

Size of carrot	Location* a	b	c	% inoculated*** sites infected	% taproots infected	Mean** diameter lesion (mm)
	% inoculated sites infected					
Small	83.3	nd	41.7	67.9	96.4	2.80 ± 1.61
Medium	75.0	50.0	60.7	58.9	89.3	3.74 ± 1.97
Large	76.9	57.7	61.5	63.5	100.0	4.41 ± 2.31

a:** agar disc on the upper root *:** uninfected sites (zero) were not included
b: agar disc on the middle root **nd:** not determined *****:** mean of a, b, c
c: agar disc on the lower root
Data are the means and standard deviations of either 35 (small and medium size) or 15 (large size) carrot roots.

3.2. THE EFFECTS OF WOUNDING, MEDIUM AND INOCULUM ON CAVITY SPOT FORMATION

To optimise the inoculum conditions *in vivo*, 4 different treatments were tested in this experiment. These were: i) Wounded + CMA (medium); ii) Wounded + V8 (medium); iii) Non wounded + CMA; iv) Non wounded + V8.

The carrot roots cv. Burton were prepared as described in Chapter 2, Section 4.2. *P. violae* was grown in CMA and V8 juice agar for 7 days at 20 ± 1 °C. To determine the effect of wounding, carrots were scratched (1 mm in depth) with a sharp blade to create a shallow cross on the surface of roots for wounding treatments. Five moist chambers containing 5 taproots were enclosed with polythene bags to increase the relative humidity and incubated in the dark at 20 ± 3 °C, 90% RH growth chamber.

The observations were taken at 2, 4 and 6 days intervals.

Table 3.17: Effects of different treatments on cavity spot formation in carrots 6 days after inoculation

Treatments	% taproots infected	% inoculated sites infected	Mean diameter * lesion (mm)
CMA + non-wounded	57.9	21.3	2.91 a
CMA + wounded	90.0	48.0	2.72 a
V8 + non-wounded	100.0	74.7	5.14 b
V8 + wounded	100.0	80.0	6.32 b

* : Uninfected sites (zero) were not included. Means within columns followed by the same letters are not significantly different according to Duncan's Multiple Range Test (P= 0.05). Each value is the mean of five replications of five carrots. Each value is mean of 25 replicate carrots (5 inoculation per carrot).

It is apparent from Table 3.17 that V8 medium inoculum resulted in greater mean percentage (77%) and mean severity (35%) of infection. The severity of infection was approximately twice greater than CMA plugs ($p < 0.01$, Appendix 16a). Watersoaked lesions were evident in 2 days with V8 but not with CMA inoculum (see Plate 10) Wounding was not required for infection implying that *P. violae* must penetrate intact periderm (i.e. the endodermis which forms the outer layer of carrot taproots). The severity of disease was affected by the time ($p < 0.01$, Appendix 16b). These results show that the most effective medium was V8 juice agar and time for determination of effective cavity spot formation was 6 days and therefore these conditions were chosen for future experiments.

3.3. THE EFFECT OF COINOCULATION OF *Ecc* AND *P. violae* ON CAVITY SPOT FORMATION

The purpose of the experiment was to examine whether *P. violae* infections facilitate entry of soft rot bacteria *Ecc* (SCRI 1039) which are unlikely to penetrate intact periderm.

The carrot roots cv. Burton were prepared as described above. *P. violae* was grown on V8 juice agar for 7 days at 20 ± 1 °C. *Ecc* was inoculated into LB broth and shaken overnight at 200 rpm at 30 °C. Twenty µl of bacterial suspension (10^9 cfu/ml) was placed on the non-wounded carrots and covered with three 5 mm plugs from *P. violae* inoculated V8 juice agar. V8 agar plugs without *P. violae* were used as control. Five moist chambers containing 5 taproots were enclosed with polythene bags to increase the relative humidity and incubated in the dark at 20 ± 3 °C, 90% RH growth chamber.

Table 3.18: Effect of coinoculation with *P. violae* on infection by *Ecc* carrots

Treatment	% taproots infected	% inoculated sites infected	Mean diameter* lesion (mm)
P. violae	100	74.7	5.14 ± 1.31
Ecc	0	0	0
Ecc* + *P. violae	100	78.7	10.0 ± 1.27

* : Soft rot symptoms observed with *Ecc* were clearly distinct from cavity spot symptoms. Uninfected sites (zero) were not included. Data are the mean and standard deviations of five replications of five carrots.

Pythium violae alone resulted in typical cavity spot symptoms on the carrot taproots (see Plate 10). There was no noticeable rotting on the roots 6 days after inoculation. Coinoculation with *Ecc* produced discoloured rotting and lesion sizes were greater than with *P. violae* alone. All carrot roots were infected when coinoculation treatment was used while 25% of inoculated sites remained uninfected when *P. violae* alone was used (see Table 3.18). Thus it can be concluded that *P. violae* facilitated the entry of *Ecc* in carrot roots.

3.4. OPTIMISATION OF CAVITY SPOT FORMATION WITH *P. violae* INFESTED SEEDS

The previous experiment results showed that when carrot roots were inoculated with V8 plugs containing *P. violae*, the percentage of the inoculum sites that resulted in infection was only *ca.* 75-80% (see Table 3.17).

In an attempt to optimise further the inoculation conditions for screening regenerated taproots with respect to *P. violae* resistance, different seeds infested *P. violae* were inoculated onto carrot roots for testing cavity spot formation.

3.4.1. Growth of *P. violae* on Different Seeds

The growth of *P. violae* on different seeds was examined to investigate which supported its growth. Hemp, rice, barley and wheat seeds were cooked for 20 min (water, 100 °C) while corn (maize) seeds were boiled for 1 hour before being left in water overnight (Anon, 1983).

Five 250 ml conical flasks containing 150-200 ml grains in each and in 4 petri dishes were set up for each growth source. Sterile filter paper moistened with 2 ml of sterile water was also placed into the petri dishes. Seeds were sterilised twice at 15 min, 1.5 bar/120 °C. Four flasks and 3 petri dishes were inoculated with 10 V8 plugs (2mm diam.) containing *P. violae.* Controls were set up with uninoculated V8 plugs. Flasks and petri dishes were incubated at 20 ± 2 °C, in the dark. Twenty three days after inoculation 20 ml sterile water was added to conical flasks and 5 ml to petri dishes to encourage further growth of the fungus. Any flasks not showing growth of *P. violae* at this stage were re-inoculated with other seeds that showed fungal growth. Growth was recorded after 22 and 35 days. A growth index was used to evaluate the development of fungus over the time (Table 3.19).

Extensive growth of *P. violae* appeared on barley, rice and corn seeds both in the conical flasks and petri dishes as shown in Plate 11. These seeds were therefore chosen for inoculation of tap roots. Growth on wheat was successful only after re-inoculation with infested barley seeds after 22 days. Hemp seeds generally showed no fungal growth except occasionally after re-inoculation.

Table 3.19: Growth of *P. violae* on different seeds

Treatments	Seed	No. of days after inoculation	Growth of *P. violae* *
Petri dishes	Hemp	22 35	0.0 2.0
	Barley	22 35	2.3 4.3
	Wheat	22 35	0.7 3.3
	Rice	22 35	1.0 4.0
	Corn	22 35	4.0 4.5
Conical flasks	Hemp	22 35	0.0 0.0
	Barley	22 35	3.5 5.0
	Wheat	22 35	0.0 4.0
	Rice	22 35	4.0 5.0
	Corn	22 35	1.8 4.8

*** The growth index used:**

0- no growth **2**- low growth **4**- high growth
1- sparse growth **3**- moderate growth **5**- extensive growth

3.4.2. Induction of Cavity Spot by *P. violae* Infested Seeds

The objective of this experiment was to determine if *P. violae* infected seeds could be more effective in producing lesion than the standard method based on agar inoculum.

Carrots (cultivar unknown) supplied freshly from a local farm were inoculated with *P. violae* infested barley, rice and corn seeds and V8 plugs. Uninoculated V8 plugs

and seeds with no fungal growth were used as controls. Four moist chambers containing 5 carrots in each treatment were placed into a growth chamber at 20 ± 1 °C, 98% RH and in the dark. Plugs and seeds were removed after 5 days and cavity spot development was measured 1 day later. The average diameter of lesions and percentage of carrots with cavity spots are presented in Table 3.20.

Table 3.20: The effect of different inoculum types in producing cavity spot on carrot

Treatments	% taproots infected	% inoculated sites infected	Mean diameter * lesion (mm)
Corn	95	51.2	6.27 a
V8	100	69.8	6.35 a
Rice	80	55.8	7.44 a
Barley	95	73.6	9.87 b

* : Uninfected sites (zero) were not included. Means within columns followed by the same letters are not significantly different according to Duncan's Multiple Range Test (P= 0.05). Each value is the mean of four replications of five carrots.

Results show that infested barley resulted in the largest cavity spot lesions (Plate 12) and was significantly different from rice, corn and V8 ($p < 0.05$, Appendix 17). Barley was also the most successful in producing lesions from 73% of inoculations. However, the percentage of the infected sites of barley was similar to V8 plugs. The percentage of the frequency of infected sites of barley was also not significantly different from V8 plugs or from rice. Rice and corn both produced just over 50% infection success even though the lesions from infested rice were larger than those with V8 inoculum.

Plate 10: Effect of different treatments on cavity spot formation in carrot roots. Note: wounding is not required:

i. ***P. violae*** **+ CMA agar + Wounded,**
ii. ***P. violae*** **+ CMA agar + non-Wounded,**
iii. ***P. violae*** **+ V8 agar + non-Wounded**
iv. ***P. violae*** **+ V8 agar + Wounded,**
v. **V8 agar + non-Wounded +** ***Ecc*** **+** ***P. violae***

Plate 11: Growth of ***P. violae*** **on various seeds. Note absence of growth on hemp seeds but extensive growth on barley and corn seeds 22 days after inoculation**

Plate 12: Inoculation of carrot taproots with ***P. violae*** **infested seeds. The most extensive symptoms resulted from** ***P. violae*** **infested barley inoculum**

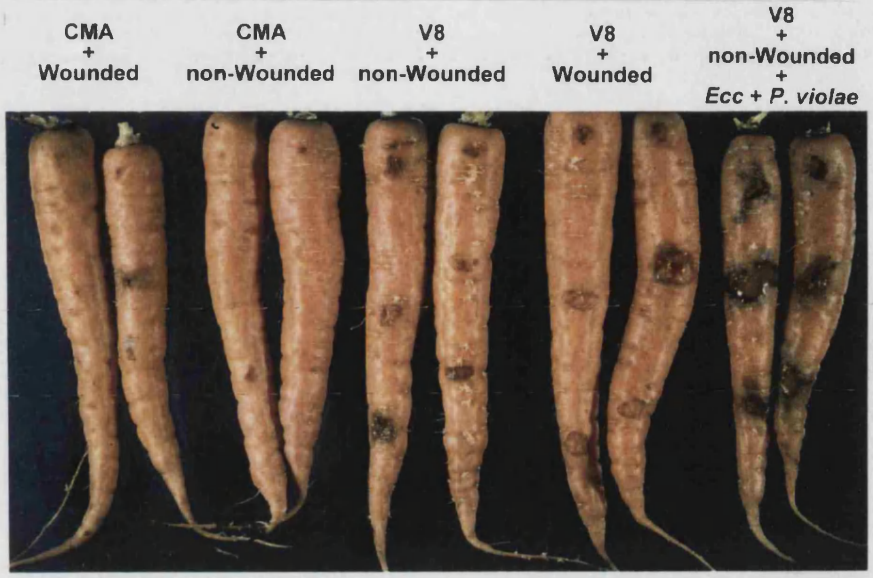


Plate 10

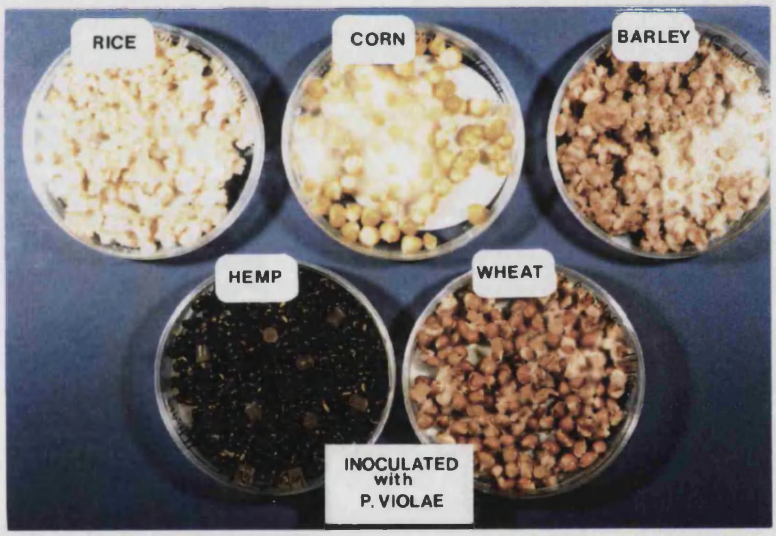


Plate 11

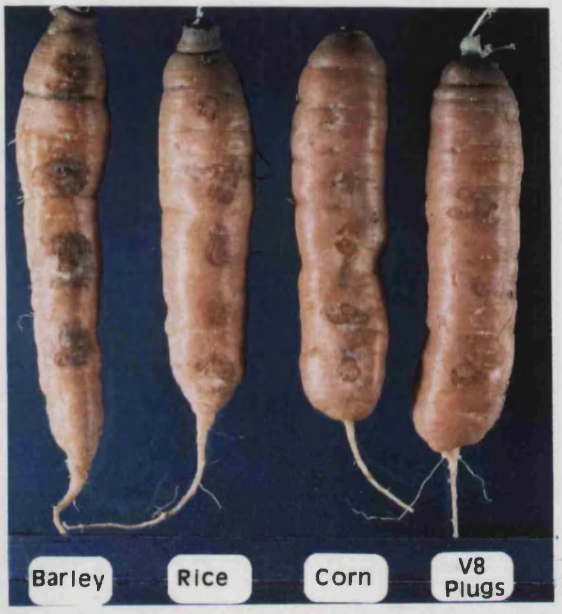


Plate 12

3.5. THE EFFECT OF PLANTING TIME ON CAVITY SPOT FORMATION

The aim of the experiment was to examine whether planting time was related to susceptibility to cavity spot formation on carrot roots.

Carrots cv. Morot Duke were sown at Bathampton Field Station (University of Bath) from February to May at monthly intervals. *P. violae* was grown as described in Materials and Methods, Section 4.1. The roots were harvested when they were 5-month-old and were inoculated at the same day at 3 (for carrots sown on February and March) and 5 (for carrots sown on April and May) different sites along the length of roots with 5 mm diameter discs containing *P. violae.* V8 plugs without *P. violae* were placed on roots as controls. Five carrot roots per moist chamber and five replicate chambers were set up for each month at 20 ± 2 °C, 98% RH in the dark. Plugs were removed after 5 days and any lesions were measured. Only the mean diameter of lesions with cavity spot and the percentage of carrots with cavity spot are presented in Table 3.21. Controls were omitted to determine the mean percentage of carrots with cavity spot.

The mean percentage of carrot with cavities was 70-95% for all months. Analysis of variance displayed that the effect of the planting time on cavity spot formation was not significant (Appendix 18).

Table 3.21: Effect of planting time on pathogenicity of *P. violae* on carrots cv. Morot Duke

Months	% taproots infected	% inoculated sites infected	Mean diameter lesion * (mm)
February	89.5	75.4	7.03 ± 1.98
March	100	95.0	7.22 ± 1.35
April	100	87.2	7.17 ± 2.14
May	100	69.8	6.35 ± 1.31

* : Uninfected sites (zero) were not included. Each value is the mean and standard deviation of five replications of five carrots.

3.6. SCREENING SOMACLONES FOR DISEASE RESISTANCE TO *P. violae*

The aim of the experiment was to regenerate plants from tissue culture techniques and to test disease resistance or tolerance to *P. violae* of regenerated taproots.

Carrot taproots were produced as explained in Chapter 2, Section 1.5. Three hundred individual carrot roots were inoculated at 3 different sites along the length of the roots with 5 mm diameter V8 plugs containing *P. violae* and placed into moist chambers (10 roots per moist chamber) at 98% RH, 20 ± 1 °C in the dark for a week. The diameter of cavity spot, the percentage of uninfected roots and percentage of inoculated sites that gave lesions were determined.

Table 3.22: Pathogenicity of *P. violae* on regenerated taproots of carrot cv. Morot Duke

Regenerated tap roots	% taproots uninfected	% inoculated sites infected	Mean diameter * lesion (mm)
Morot Duke (Original seed line)	0	94.2	9.0 ± 1.8
Taproots regenerated from Morot Duke	6.1	76.2	9.2 ± 2.5

* : Uninfected sites (zero) were not included. Data are the mean and standard deviations of 300 individual regenerated roots.

All the inoculated roots obtained from the original seed line were susceptible to cavity spot while 6.1% of the taproots regenerated from Morot Duke was free from cavity spot. Although there was reduction on the percentage of inoculated sites that gave lesions, the severity of disease was the same on both regenerated roots and roots from original seed line (see Table 3.22).

3.7. PRODUCTION OF CELL WALL DEGRADING ENZYMES BY *P. violae*

The production of cell wall degrading enzymes by pathogens and the role of such enzymes in pathogenesis and tissue maceration have been the major target of many investigations. The features of symptoms induced by phytopathogenic soft rot *Erwinia, Pseudomonas* and *Pythium* spp. suggest that the production of pectic enzymes might be important components in pathogenesis (Salmond, 1994; Winstead and McCombs, 1961). Although reports has been found concerning enzyme production by *P. violae*, a number of extracellular enzymes including both pectinolytic enzymes and cellulases have been detected *in vivo* and *in vitro* from *Pythium* spp. (Deacon, 1979; Elad et al., 1985; Endo and Colt, 1974; Janardhanan and Husain, 1974; Mellano et al., 1970; Nemec, 1974; Sadik et al., 1983; Winstead and McCombs, 1961; Wood and Gupta, 1958).

Against this background of enzyme production by *Pythium* spp., it was decided to investigate the involvement of cell wall polysaccharide degrading enzymes produced by *P. violae in vivo* and *in vitro* in relation to pathogenesis.

3.7.1. Determination of the Optimum Media for Growth and Enzyme Production

Three different media were tested in order to obtain optimum growth and study the *in vitro* production of cell wall degrading enzymes by *P. violae*:

1. V8 juice broth medium (Baker and Bateman, 1978);

2. DowElanco medium (DowElanco, Letcombe Labs, Dr. P.F.S. Street pers. comm.);

3. Wood and Gupta medium (Wood and Gupta, 1958).

Static and shake cultures were tested to establish which culture method would result in the greatest mycelium production.

Ten plugs taken with a No. 2 cork borer from the edge of 7-day-old CMA and V8 cultures were used to inoculate both shake and static cultures. For shake cultures, 250 ml conical flasks and for static cultures 200 ml medical flats containing 100 ml and 40 ml of the above media respectively were set up with either V8 (2 replicates) or CMA (5 replicates) inoculum. Conical flasks were incubated in the 150 rpm shaker at 20 °C. Static cultures were lain on their side with the tops loosened and incubated at 20 °C.

The mycelia were collected by filtration through fine muslin and transferred to a pre-weighed aluminium foil cup after 11 days. This was then dried overnight at 60°C before measurement of dry weight.

Growth in static culture produced a mycelial mat, but in shake culture produced dispersed mycelial growth, mainly around the agar inoculation plugs, which was easily recovered for growth analysis.

P. violae produced higher mycelial mass in the DowElanco medium (Table 3.23). Shake cultures resulted in greater dry weight of *P. violae* than static cultures. The higher amount of growth occurred in either static or shake cultures inoculated with V8 plugs.

DowElanco medium and shake cultures were chosen for the future experiments as these encouraged extensive mycelial growth and maximised contact of fungus with insoluble carbon sources added into the medium i.e. suberin and carrot cell walls.

Table 3.23: Growth of *P. violae* in 3 different media in static and shake culture from two inoculum sources

	Static culture Mean dry weight (mg/40 ml)		Shake culture Mean dry weight (mg/100 ml)	
	Inoculum		Inoculum	
Media	**CMA**	**V8**	**CMA**	**V8**
Baker and Bateman	157 ± 22.9	58 ± 56.6	186 ± 42.3	316
Wood and Gupta	122 ± 4.33	200 ± 11.3	254 ± 37.3	276 ± 17.0
DowElanco	235 ± 17.3	232 ± 36.0	359 ± 35.6	379 ± 43.8

Each value is the mean and standard deviation of two replications for V8 inoculations and five replications for CMA inoculations. **V8** :V8 juice agar **CMA** :Corn meal agar.

3.7.2. Detection of Cell Wall Degrading Enzymes by *P. violae in vitro*

3.7.2.1. Growth of *P. violae* on Different Carbon Sources

P. violae was grown on DowElanco medium supplemented with 4 different carbon sources in order to determine regulation of enzyme synthesis. Carrot cell walls, pectin (Sigma Chemicals) and carboxymethly cellulose (BDH) were used as potential inducers and which should not effect catabolite repression because of their insolubility or degree of polymerisation respectively, and glucose was added to provide non inducing and repressing conditions. All the media contained 1% (w/v) of the appropriate carbon source.

One hundred ml of each medium (pH 6.0) in 250 ml conical flasks were inoculated individually using aseptic techniques with ten, 5 mm agar plugs taken from the edge of V8 plates using a cork borer and flasks were incubated in a rotary shaker (160 rpm) at 20 °C, in the dark. Three replicates were produced for each carbon source. 20 ml samples of culture fluids were taken aseptically from each flask at 9, 11 and 16

days after inoculation. The samples were then centrifuged (≤ 30 min, 13000×g) to remove the mycelial and substrate debris.

The growth of *P. violae* on the carbon sources used was observed at several intervals during the *in vitro* cultivation of the fungus. Growth was estimated with the use of a growth index and finally by the measurement of fresh weight of the mycelia (except this was not possible on insoluble cell walls).

Table 3.24: Growth of *P. violae* on different carbon sources

Carbon source	Days					Fresh weight g/100 ml
	2	4	7	9	14	
Glucose	2	3	4	4	4.7	1.39 a
Cell walls	1	1	1	1	2	nd
Pectin	1	1	1	2	2	0.26 b
Cellulose	1	1	2	2	2	0.22 b

Growth index: 0-No growth, **1**-Mycelia just evident on the agar plugs, **2**-Substantial growth but not covering the whole plug, **3** → **5** -Moderate → extensive growth. Means within columns followed by the same letters are not significantly different according to Duncan's Multiple Range Test (P= 0.05). Each value is the mean of three replications. **nd** :not determined.

There were significant differences between growth on the carbon sources used (Table 3.24) ($p < 0.01$, Appendix 19). Maximal growth occurred on glucose although mycelium was mainly around the V8 agar plugs. Growth on cellulose and pectin was similar but much less than on glucose.

Growth was not observed to any great extent on cell walls as a sole carbon source, but sparse fungal growth was evident around the sides of the conical flasks.

3.7.2.2. Production of Polygalacturonase, Pectin lyase and Cellulase by *P. violae* *in vitro*

The objective of the experiment was to examine the production of cell wall degrading enzymes by *P. violae* when grown in culture on different carbon sources,

which may function as inducer of production. Of the various wall degrading enzymes produced, only endo-polygalacturonase, pectin lyase and cellulase were studied because of their likely involvement in pathogenesis, as evidenced by synthesis *in vitro* and *in vivo* by other *Pythium* spp. (Endo and Colt, 1974; Winstead and McCombs, 1961; Wood and Gupta, 1958).

The experiment was set up as in Section 3.7.2.1 and the samples were assayed for enzyme activities as in Chapter 2, Section 6.

Table 3.25: Production of cell wall degrading enzymes by *P. violae* on different carbon sources *in vitro*

	Enzyme activity								
Carbon Sources	**PL[a]**			**endo-PG[b]**			**Cellulase[c]**		
	Days								
	9	**11**	**16**	**9**	**11**	**16**	**9**	**11**	**16**
Pectin	0	0	0	0	0	0	7.88	25.32	52.57
Cellulose	10.7	3.67	5.18	0	0	0	64.1	91.27	111.4
Glucose	0	0	0	0	0	0	26.1	35.0	87.1
Cell walls	0	0	0	0	0	0	0	0	38.24

[a] PL activity was assayed by UV absorbance at 240 nm and shown as nkat
[b] endo-PG activity was assayed viscometrically and is expressed as RVU
[c] Cellulase activity was assayed viscometrically and is expressed as RVU

P. violae did not produce endo-PG on any carbon source. No PL was detected in cultures containing carrot cell walls, pectin or glucose as a sole carbon source but activity was detected in all samples from cellulose as carbon source. Highest extracellular PL activity occurred by (or before) 9 days, then activity decreased with time (Table 3.25). Cellulase activity increased markedly with time on all carbon sources. Cellulase activity of culture filtrates from 16-day-old cultures was much greater than that of 9- and 11-day-old cultures. Highest levels were on cellulose, glucose, pectin and

carrot cell walls respectively. On the latter, activity only appeared by day 16 in carrot cell walls and may reflect the poor growth rate of *P. violae* on this substrate.

3.7.2.3. Extraction for Cell-Bound Associated Activities

The objective was to detect activity remaining associated with the mycelial surface. Cell bound enzymes may play a key role in host penetration but do not appear in the more facile analysis for extracellular enzymes.

The mycelia grown in media containing pectin, cellulose, glucose and cell walls as carbon sources were removed from the fluids and washed with a buffer solution (0.02 M phosphate buffer containing 0.4 M sodium chloride pH 6). This was designed to desorb any enzyme ionically bound to the mycelial surface. Mycelium collected from each conical flask was placed into 250 ml conical flasks containing 20 ml of buffer solution and rotated in a shaker for 1 hour. Mycelia were than removed and supernatants assayed for enzyme activities.

Table 3.26: Cell associated enzyme activities from *P. violae*

Carbon Sources	Enzyme activity		
	PL[a]	endo-PG	Cellulase
Pectin	7.726	0	0
Cellulose	2.223	0	0
Glucose	0	0	0
Cell walls	0	0	0

[a] PL activity was assayed by UV absorbance at 240 nm and shown as nkat.

No endo-PG activity could be detected in any of the buffer washes. Attempts to detect PL in the buffer washes from the mycelium from glucose and cell wall cultures also gave negative results. However, PL was found in the washes from the mycelia

grown on cellulose and pectin as the sole carbon sources although activity on cellulose was lower than that found in cell-free fluids (see Table 3.26).

Cellulase activity was detectable but it could not be measured accurately over a reasonable period of time. Thus, cellulase appears to be almost entirely extracellular but PL is entirely cell-bound in pectin grown cultures in contrast to extracellular activity from cellulose cultures (Table 3.25).

3.7.2.4. Growth of *P. violae* on Different Carbon Sources for Suberinase Production

Suberinase catalyses the hydrolysis of an insoluble biopolyester, suberin, which is found in plant roots and around storage organs such as carrot tap roots. *P. violae* enters unwounded taproots of carrot and therefore must penetrate outer suberized endodermis. This might require degradation of suberin. Therefore attempts were made to detect any suberinase produced by *P. violae in vitro* culture conditions.

DowElanco medium containing 3 different carbon sources were tested to determine the growth of *P. violae*. The carbon sources used were suberin (0.5%), suberin and sucrose (0.5%, 0.075% respectively) and sucrose (0.075%). Fifty ml of above media was added to 150 ml conical flasks and inoculated with five, 5 mm agar plugs from the growing margin of *P. violae* on V8 agar. Four conical flasks were set up for each treatment and incubated at 20 ± 1 °C in a shaker at 160 rpm. The sucrose in the suberin and sucrose alone were intended as a starter to initiate mycelial growth, after which suberin might be degraded. The control used for growth observations was sucrose at 0.075%.

Shake culture was used to ensure contact between the suberin (which would settle to the bottom of the flasks) and the fungus. A growth index was used to evaluate the development of fungus over the time.

P. violae grew extremely sparsely on the medium containing suberin as the sole carbon source (Table 3.27). Some growth occurred in suberin with low sucrose but it was very difficult to assess growth in either of the suberin containing media as the suberin obscured the agar plugs around which most growth occurred. The highest growth was found in low sucrose alone suggesting that suberin has some inhibitory effect on growth.

Table 3.27: Growth of *P. violae* on suberin, suberin and low sucrose and sucrose

Carbon source	Days					
	3	5	7	10	13	17
Suberin	0	1	1	1	1	1
Suberin and low sucrose	0	1	1	1	1	1
Low sucrose	1	2	2	2	2	2

Growth index:
0: no growth
1: growth just detectable from inoculation plugs
2: sparse growth

3.7.2.5. Production of Suberinase by *P. violae in vitro*

An experiment was set up as explained above and the samples were assayed for enzyme production as described in Materials and Methods, Section 6.3.

One ml samples were taken after 3, 5, 7, 10, 13 and 17 days and were centrifuged at 11500×g for 10 minutes before being assayed for suberinase.

The amount of suberinase production increased over time, peaking after 13 days of incubation in the suberin and low sucrose media and steadily increasing in suberin medium (Table 3.28). The rates of suberinase production in the two media were similar for the first 10 days of the experiment which indicates that suberinase production was not repressed by the low sucrose supplement.

Table 3.28: Suberinase by *P. violae in vitro*

Incubation time (days)	Suberinase activity (nkat)	
	Suberin	Suberin and low sucrose
3	11.65	14.72
5	9.79	16.16
7	11.53	15.74
10	13.09	15.98
13	15.74	54.17
17	20.06	23.6

Suberinase activity was assayed by UV absorbance at 405 nm.

3.7.3. Determination of Cell Wall Degrading Enzymes Produced by *P. violae in vivo*

The objective was to investigate if and when cell wall degrading enzymes were produced *in vivo.*

The lesions produced on the carrot roots by *P. violae* were excised 2, 4, 6 and 7 days after inoculation of carrot roots. These samples were stored at -70 °C until needed. Uninoculated tissues were used as controls. Enzymes were extracted from tissues containing cavity spots and assayed as before.

Table 3.29: Determination of polygalacturonase and cellulase produced by *P. violae in vivo*

	Enzyme activity			
	endo-PG[a]		Cellulase[b]	
Time (days)	Inoculated tissue	Uninoculated tissue	Inoculated tissue	Uninoculated tissue
2	0	0	0	0
4	0	0	0	0
6	0	0	8.85	0
7	0	0	6.45	0

[a] endo-PG activity was assayed viscometrically and is expressed as RVU
[b] Cellulase activity was assayed viscometrically and is expressed as RVU

Polygalacturonase activity was not detected in any of the infected tissues while cellulase activity was found in samples from the oldest lesions. No uninfected tissue showed any enzyme activity. This suggests that cellulase activity is likely to be of fungal origin (see Table 3.29).

4. SCREENING SOMACLONES FOR DISEASE RESISTANCE TO *Erysiphe heraclei*

Hypocotyl explants from commercial, open pollinated, new red intermediate called NRI-92 (Suttons) were dissected and put into MS2 supplemented with 5μM 2,4-D media to induce embryogenic callus formation for two weeks. Calli were then transferred into MS2 media for production of plantlets. Plantlets were acclimatised as described in Chapter 2, Section 1.5. One hundred and sixty randomly chosen somaclones were planted into 12.5 cm high, 10.5 cm diam. pots and were assessed for susceptibility to powdery mildew. Plantlets were inoculated when 10-12 weeks old by dusting conidia over leaves, then incubated at *ca.* 25 °C. Two to three weeks after inoculation, typical symptoms of powdery mildew caused by *E. heraclei* were seen on the leaves and stems (Plate 13). A disease index was used to evaluate disease susceptibility (see Table 3.30).

Table 3.30: Resistance to *E. heraclei* of regenerated somaclones

Disease susceptibility*	**0**	**1**	**2**	**3**	**4**
Regenerated plants (% affected)	26.7	13.6	25.8	21.2	12.7

* **Disease index:** **0** : No symptoms; **1** : 25% of the leaf area covered with mildew **2** : 25-50% of the leaf area covered with mildew **3** : 50-75% of the area covered with mildew **4** : 75-100% of the leaf area covered with mildew.

A high proportion of regenerated somaclones (mean frequency value of 26.7%) was resistant to powdery mildew; no symptoms were apparent on the leaves. This is in marked contrast to frequencies of somaclonal variants with a particular trait selected at the plant level that are estimated to range from 0.2 to 3% (e.g. Daub, 1986; Larkin and Scowcroft, 1981).

4.1. DETERMINATION OF PATHOGENICITY OF POWDERY MILDEW ON REGENERATED, SUSCEPTIBLE AND RESISTANT SEEDLINGS

To determine whether *E. heraclei* resistance was inherited by progeny, seeds were produced from the regenerated resistant somaclones and the resulting seedlings from two of them (Somaclone-4 and 15) retested for disease resistance. Also seeds from genotypes with claimed different levels of resistance to powdery mildew (see Materials and Methods, Section 1.1), were planted with the seeds from regenerants to compare disease reaction.

Two half trays containing 18 seeds for each treatment were set up. Six-week-old seedlings were inoculated with similar amounts of powdery mildew conidia (Chapter 2, Section 5.1) and incubated at 25 ± 1 °C in the green house (natural daylight) and the plants were observed after 10 days for disease symptoms. Plants were scored for disease symptoms according to scale 0 to 4 (see Table 3.31). Disease incidence was also assessed as number of leaves showing disease symptoms/total number of inoculated leaves.

The number of plants infected with powdery mildew and severity of the powdery mildew per plant were clearly reduced on somaclones regenerated from callus culture. Both somaclones showed less severe infection than plants from the original seeds (NRI 92) as can be seen in Table 3.31 and Plate 14.

Disease incidence of Somaclone-4 was significantly lower ($p < 0.01$, Appendix 20a) in comparison with the other seedlings, thus it indeed appeared virtually resistant to powdery mildew. The original seed line (NRI-92) from which the somaclones were derived showed obvious susceptibility to powdery mildew. Cultivar Danro was highly susceptible as reported by Lebeda and Coufal (1987).

Table 3.31: Pathogenicity of powdery mildew on seedlings from two regenerated lines and on susceptible and resistant seed lines

Genotype	Disease incidence (%)	Disease severity* (score)	Inoculum density spores/cm^2
Somaclone-4	9.2 a	0.3 a	87.7
Somaclone-15	32.6 b	0.8 a	79.1
NRI-92 (Original seed)	61.3 c	1.7 a	96.4
Guerenda (Susceptible)	59.3 c	1.6 b	82.9
Bertina (Susceptible)	59.3 c	2.4 b	92.8
Gavrilovskaja (Resistant)	44.7 b	1.1 b	112.7
Erstling (Resistant)	64.0 c	1.7 b	49.6
Danro (Susceptible)	68.4c	2.3 b	79.4

*** Disease index : 0 :** No symptoms; **1 :** 25% of the leaf area covered with mildew **2 :** 25-50% of the leaf area covered with mildew **3 :** 50-75% of the area covered with mildew **4 :** 75-100% of the leaf area covered with mildew.
Means within columns followed by the same letters are not significantly different according to Duncan's Multiple Range Test (P=0.05). Each value is the mean of two replications of 18 seedlings.

Disease severity was significantly higher on Guerenda, Bertina, NRI-92, Erstling and Danro leaves ($p < 0.05$) compared to the somaclones Somaclone-4, Somaclone-15 and Gavrilovskaja. Disease incidence (infected leaves/total leaves per plant) was also significantly lower ($p < 0.01$, Appendix 20b) on Soma-4 (9.2%) followed by Soma-15 (33.6%) and Gavrilovskaja (44.7%) compared to other genotypes.

Plate 13: Typical symptoms of powdery mildew on carrot cv. NRI-92 (susceptible, on the right) and Somaclone-4 (resistant, on the left) leaves 10 days after inoculation with *E. heraclei*

Plate 14: Different levels of resistance to powdery mildew of 4 cultivars and 2 regenerated plants 10 days after inoculation with *E. heraclei*:

a	b	c
Bertina	Gavrilovskaja	Erstling
NRI-92	Somaclone-15	Somaclone-4
d	e	f

Plate 13

Plate 14

4.2. DETERMINATION OF THE LEVEL OF RESISTANCE TO *E. heraclei* INFECTION IN REGENERATED, SUSCEPTIBLE AND RESISTANT SEEDS

The purpose of this experiment was to understand the source of variation and to determine the level of resistance in original seeds (NRI-92) and somaclone-4 seeds which were developed from NRI-92. One of the highly susceptible carrot cultivars, Danro, was also used as a comparison.

Four half trays for each treatment were set up. Two-month-old seedlings were inoculated mechanically or naturally with powdery mildew conidia as described in Chapter 2, Section 5.1 and incubated at 25 ± 1 °C in the green house (natural day light) and the plants were observed after 10 days for disease symptoms.

Disease incidence of each population and disease severity of each plant in the population were both assessed and the mean disease severity for each population was calculated. Plants were scored for disease symptoms according to scale 0 to 4 (see Table 3.32).

Table 3.32: The level of *E. heraclei* infection in inoculated susceptible (NRI-92), resistant (Somaclone-4) and highly susceptible (Danro) carrot plants

	Mechanical infection			Natural infection		
Genotype	**Disease incidence (%)**	**Disease severity (score)**	**Infection free plants (%)**	**Disease incidence (%)**	**Disease severity (score)**	**Infection free plants (%)**
Soma-4	9.8 a	0.5 a	79	8.6 a	0.3 a	85
NRI-92	44.2 b	1.7 b	24	63.4 b	2.4 b	15
Danro	70.3 c	2.8 b	7	86.4 c	3.5 c	0

***Growth index :** **0 :** No symptoms; **1 :** 25% of the leaf area covered with mildew **2 :** 25-50% of the leaf area covered with mildew **3 :** 50-75% of the area covered with mildew **4 :** 75-100% of the leaf area covered with mildew.

Means within columns followed by the same letters are not significantly different according to Duncan's Multiple Range Test (P=0.05). Each value is the mean of four replications of 50 seedlings.

Disease incidence and disease severity were significantly reduced from the levels on cv. NRI-92 in Somaclone-4 populations in both the mechanically inoculated and naturally infected seedlings ($p < 0.01$, Appendix 21a, b, c, d). Also, the percentage of infected plants in the Somaclone-4 populations was very low (21%). These results confirm that Somaclone-4 is relatively resistant to powdery mildew infection. Disease symptoms become severe on the susceptible line NRI-92 as well as on Danro after 10 days following both inoculation methods.

However, 15-24% of NRI-92 remained uninfected showing that open pollinated NRI-92 has a high level of disease resistance within the seeds; this contrasts with Danro which showed no disease free seedlings when challenged by natural infection.

5. SCANNING ELECTRON MICROSCOPY OF POWDERY MILDEW ON A SUSCEPTIBLE (NRI-92) SEEDLINGS AND REGENERATED (SOMACLONE-4) PLANTS

Erysiphe heraclei was studied with scanning electron microscopy (SEM) in order to compare the early stages of fungal development on the leaf surface of Somaclone-4 which showed some resistance to powdery mildew and on susceptible NRI-92 leaves derived from original seeds.

Individual NRI-92 and Somaclone-4 plants were inoculated with young conidia ($\leq$ 24 hrs) and were incubated at 25 $\pm$ 1 ^{o}C, in a greenhouse (see Chapter 2, Section 5.1). Pieces of the terminal leaflet of the youngest expanded leaf were taken 5 and 15 hrs after inoculation of plants and were frozen in liquid nitrogen, coated with gold and examination on a cryostage in the microscope.

The number of germinated conidia, conidia which formed only germ tubes, germ tubes with appressorium and elongating secondary hypha (ESH) were assessed. It is apparent from Table 3.33 that the proportion of germinated conidia was less at 5 hrs on

Somaclone-4 than on NRI-92. However, the number of germinated conidia was very similar at 15 hrs. Fungal structures formed were also similar on both Soma-4 and NRI-92 (Plates 23, 24). After 15 hrs, the initial germ tube grew extensively and up to two germ tubes per conidium were seen on Somaclone-4 inoculated leaves (Plate 24). Conidia germinating on the leaf surface formed either short or long germ tubes with lobed shaped appressoria by 5 hrs after inoculation (Plates 15-26). Almost the same frequency of appressorium formation was found on both NRI-92 and Somaclone-4 (Table 3.33). Occasionally epidermal cells underlying appressoria appear to have collapsed and this was specific to NRI-92 (Plates 18, 21). Most appressoria formed over junctions of epidermal cells (Plates 16, 18, 21, 23, 24). Appressoria also formed on the leaf hairs of Soma-4 (Plate 22). No penetration through the stomates was observed (Plates 16, 18-23).

The mycelium of the fungus developed extensively. On NRI-92 leaves, the fungus became well established as reflected by the formation of secondary hyphae by 5 hrs while Somaclone-4 had fewer secondary hyphae (Table 3.33). Secondary hyphae of *E. heraclei* on both carrot lines resulted from lateral branches from the primary appressoria (Plates 27, 28).

The poles of the conidia appeared smooth (Plates 16, 19, 21) whereas the surface of the conidia was highly convoluted in appearance (Plates 15, 16, 18, 20-25).

Table 3.33: Spore germination appressorium formation and elongating secondary hyphae by *E. heraclei* on resistant (Soma-4) (A) and susceptible (NRI-92) (B) leaves of carrot 5 and 15 hrs after inoculation

(A)

Somaclone-4				
Time (hours)	**Germinated conidia (%)**	**Germ tubes without appressoria (%)**	**Appressorium formation (%)**	**Elongating secondary hyphae (%)**
5	70.9	9.9	60.6	0.5
15	90.7	9.3	79.9	1.6

(B)

NRI-92				
Time (hours)	**Germinated conidia (%)**	**Germ tubes without appressoria (%)**	**Appressorium formation (%)**	**Elongating secondary hyphae (%)**
5	90.5	14.9	70.3	5.4
15	83.9	14.8	69.1	nd

Almost 200 conidia for Soma-4 and 150 conidia for NRI-92 were counted.
nd: not determined.

Plates 15-28: Scanning electron micrographs of powdery mildew on leaves of carrot cv. NRI-92 and Somaclone-4 (Soma-4)

All samples were taken 5 hrs or 15 hrs after inoculation of leaves.

Gun potentials up to 5, 10, 15 kV were used.

Abbreviations used in plates are,

C: conidium

A: appressorium

L: leaf epidermal cells

GT: germ tube

ST: stomate

ESH: elongating secondary hypha

LH: leaf hairs

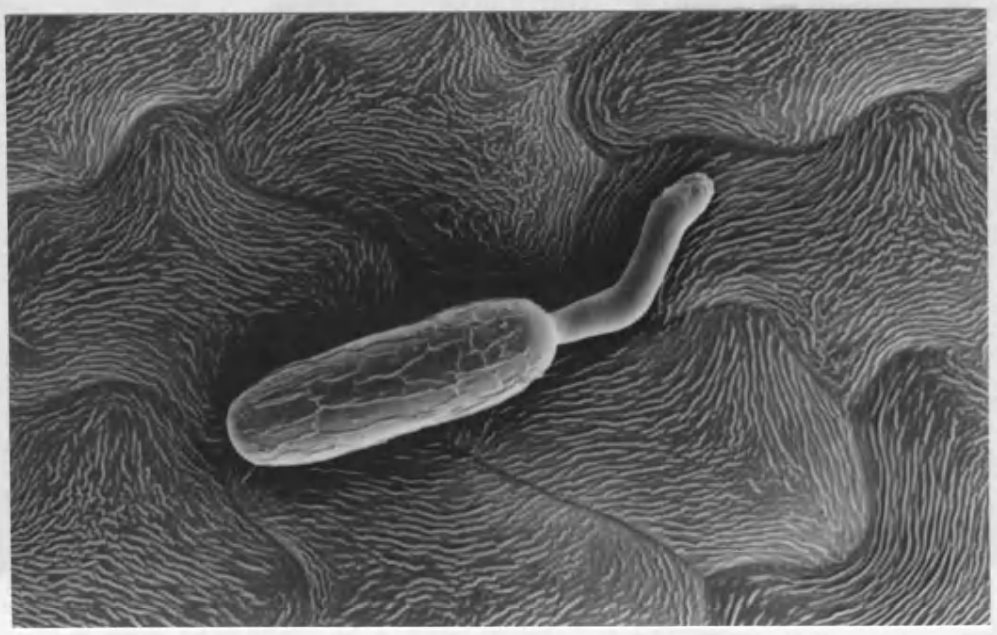


Plate 15: Germinating conidium of *E. heraclei* on cv. NRI-92, 5 hours after inoculation. Single germ tube has not formed an appressorium but has not reached the edge of epidermal cells. Note: the cuticular ridges on the epidermal cells (×2080)

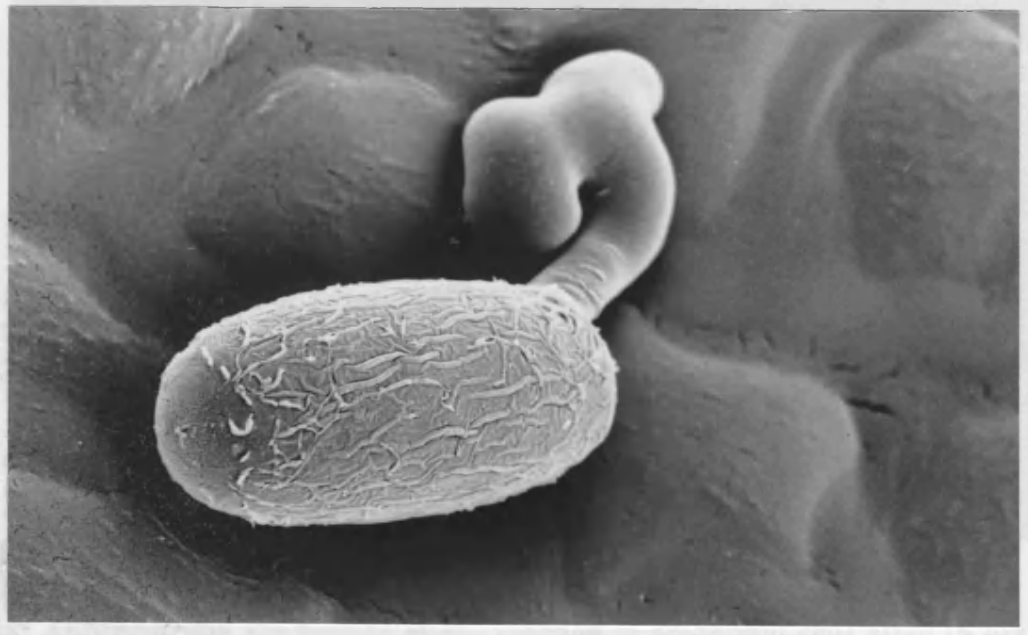


Plate 16: Conidium with single germ tube and terminal lobate appressorium formed over junction of epidermal cells on cv. NRI-92, 15 hrs after inoculation. Note: the rough ornate surface and the smooth polar end of the conidium (×4150)

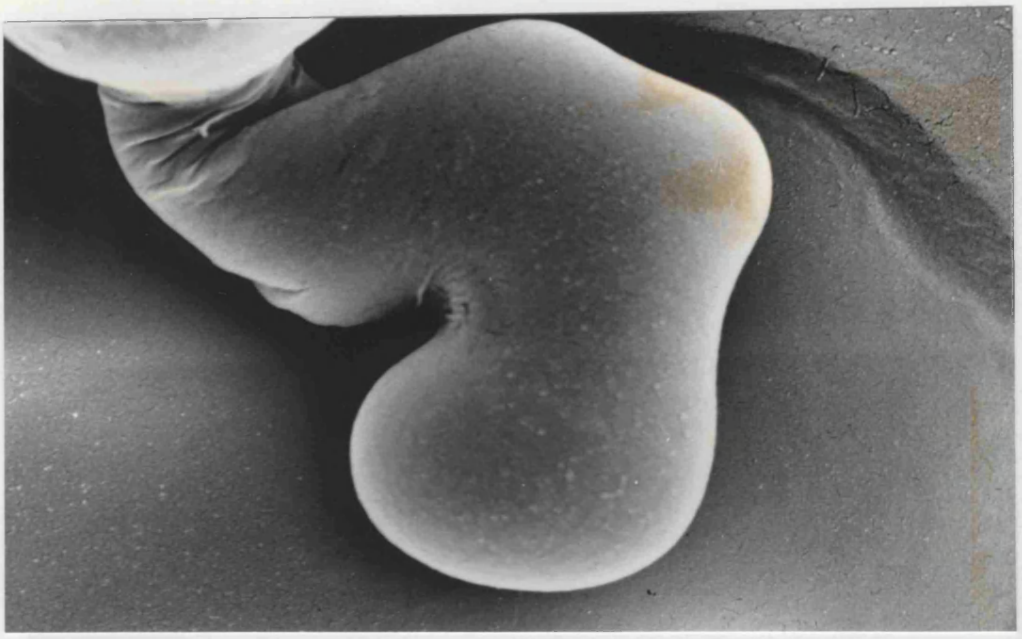


Plate 17: Detail of an appressorium cv. NRI-92. Note: lobate structure of appressorium (×10390)

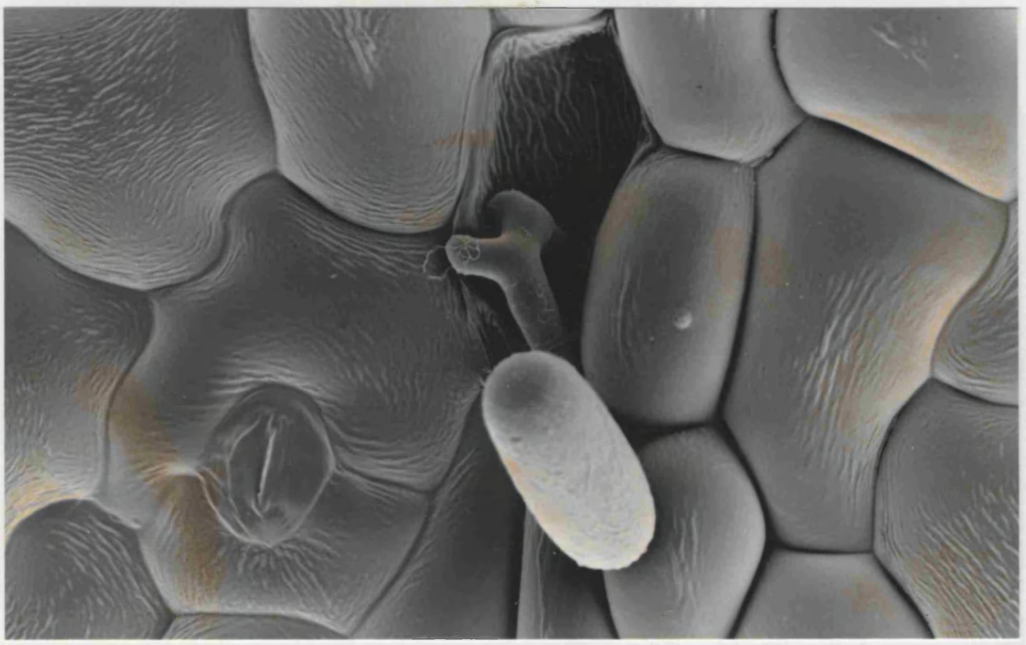


Plate 18: Appressorium are formed at the junction of epidermal cells after 15 hrs on cv. NRI-92. Growth towards and penetration of stomates was never seen. Underlying appressoria, epidermal cells appears to have collapsed (×1870)

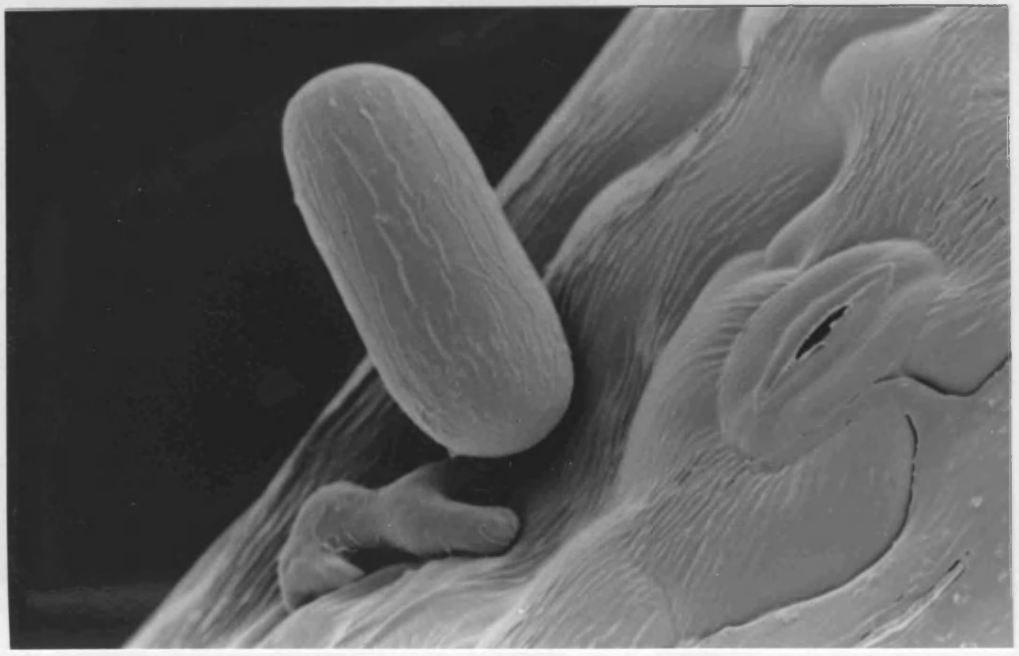


Plate 19: Description as for Plate 18 (×2910)

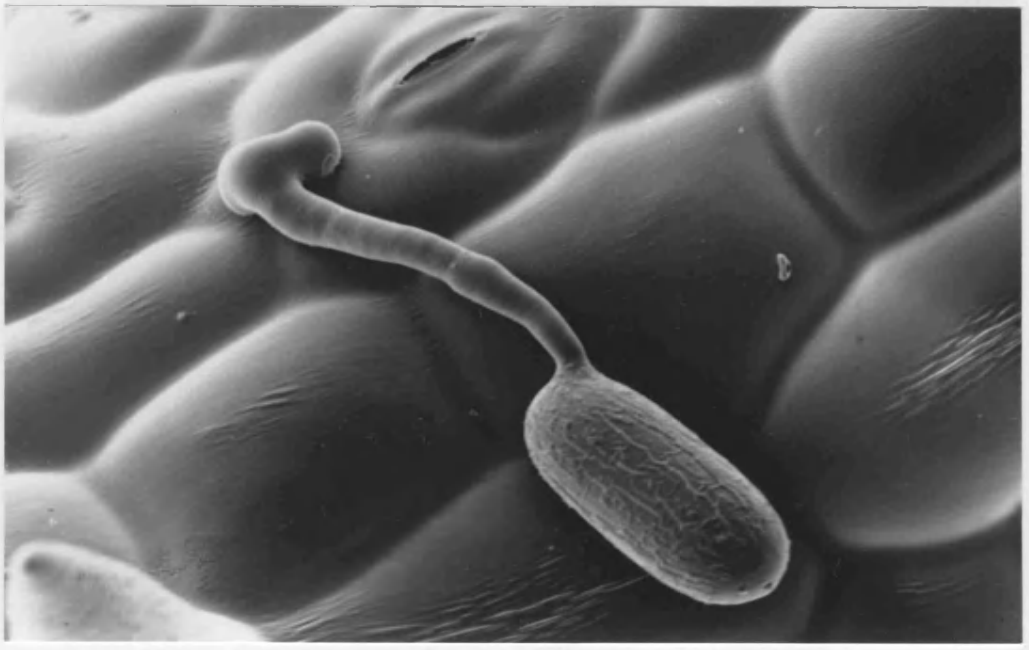


Plate 20: Germinating conidium with long germ tube and terminal lobed appressorium on the leaf of Soma-4, 15 hrs after inoculation (×2290)

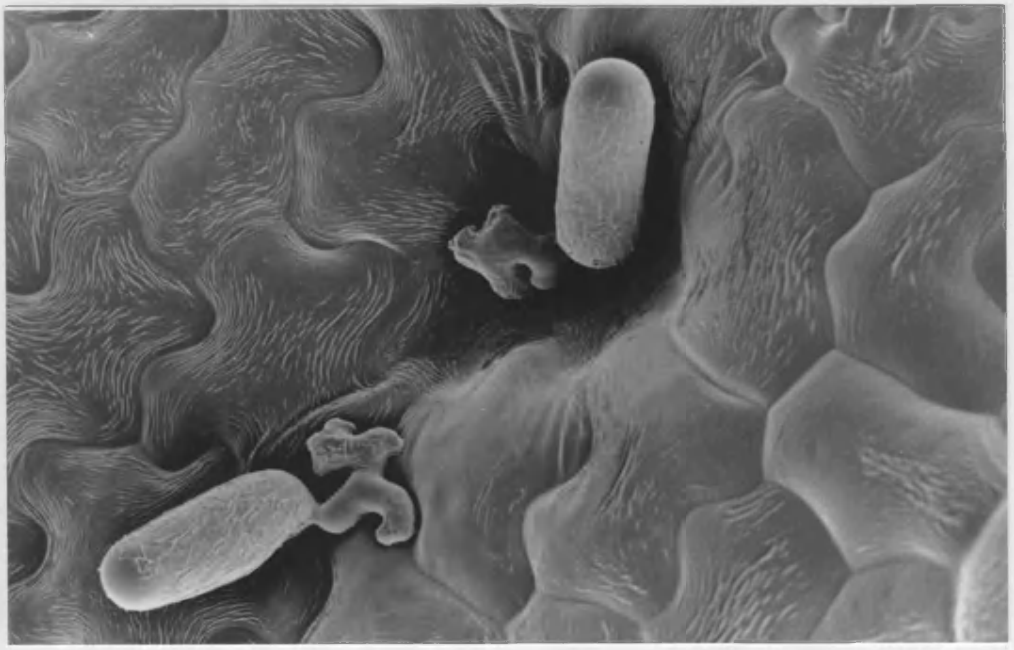


Plate 21: Two germinating conidia with short germ tubes and multiply lobed appressoria on cv. NRI-92, 15 hrs after inoculation. One underlying epidermal cell appears to have collapsed (×1450)

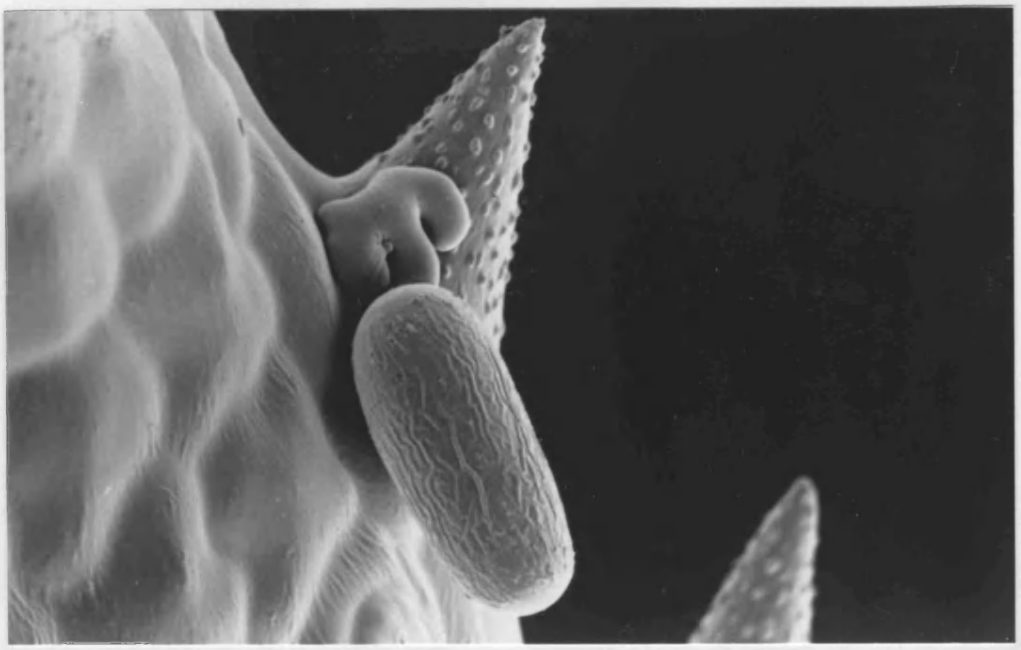


Plate 22: Germinating conidium on a leaf hair of Soma-4, 15 hrs after inoculation (×2290)

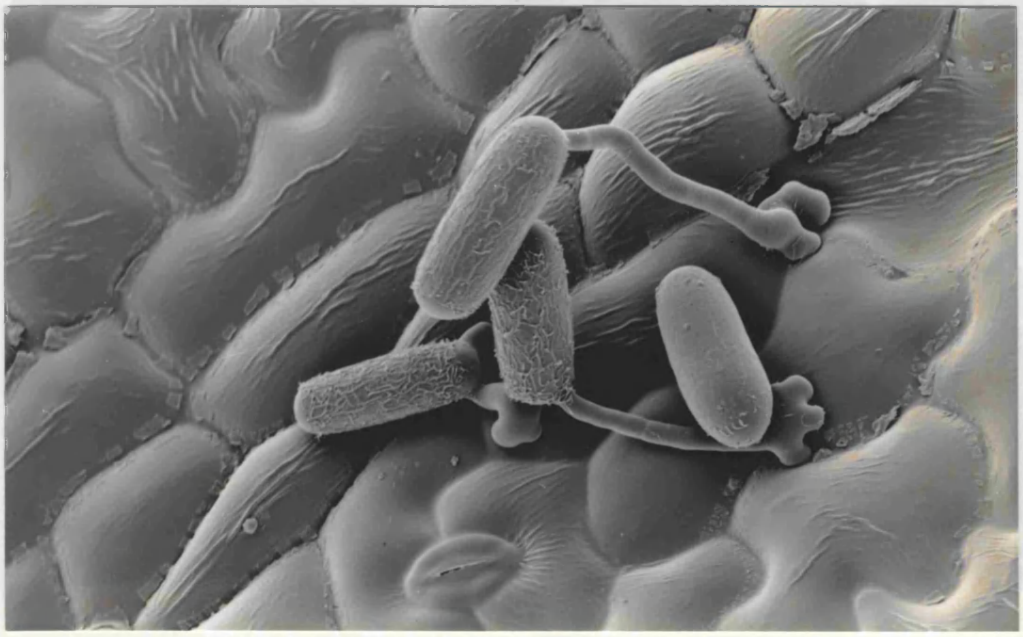


Plate 23: Development of powdery mildew on cv. NRI-92, 15 hrs after inoculation. One ungerminated conidium and 3 germinating conidia with lobate appressoria (×1250). Note residual ice crystals on part of leaf

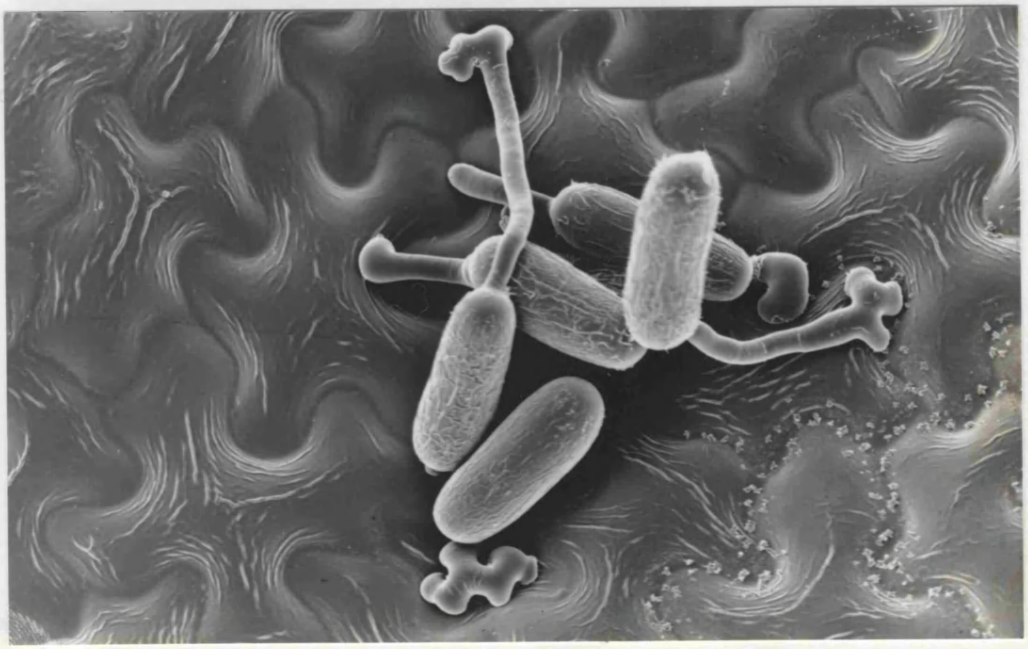


Plate 24: Fungal structures formed on Soma-4 are similar to those on NRI-92 (Plate 23). One of 5 germinating conidia has two germ tubes (×1250). Note residual ice crystals on part of leaf

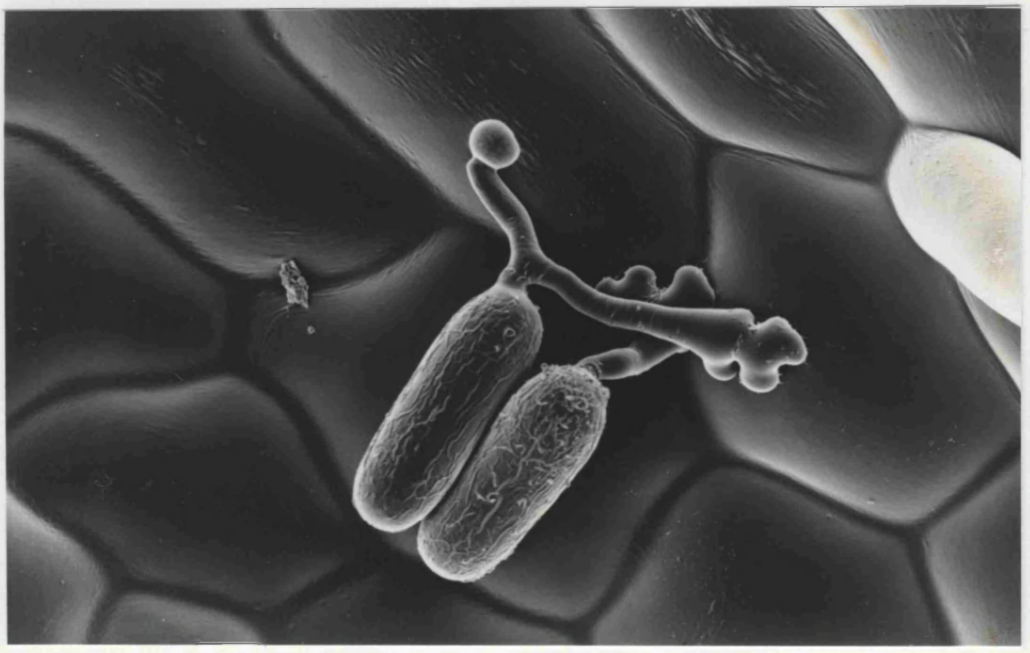


Plate 25: Bifurcating germ tube or elongating secondary hypha of a conidium on leaf surface of Soma-4, 15 hrs after inoculation. Note spherical structure at the end of second germ tube (×1660)

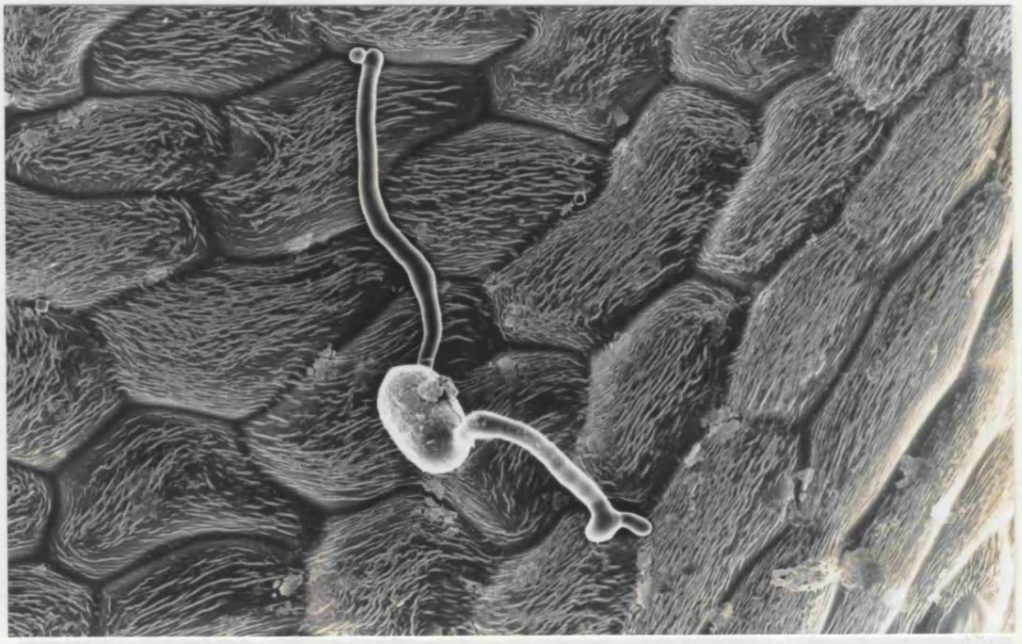


Plate 26: Germinating conidium with two germ tubes on cv. NRI-92, 15 hrs after inoculation. Note elongating secondary hypha may be arising from appressorium of one of them (×1040)

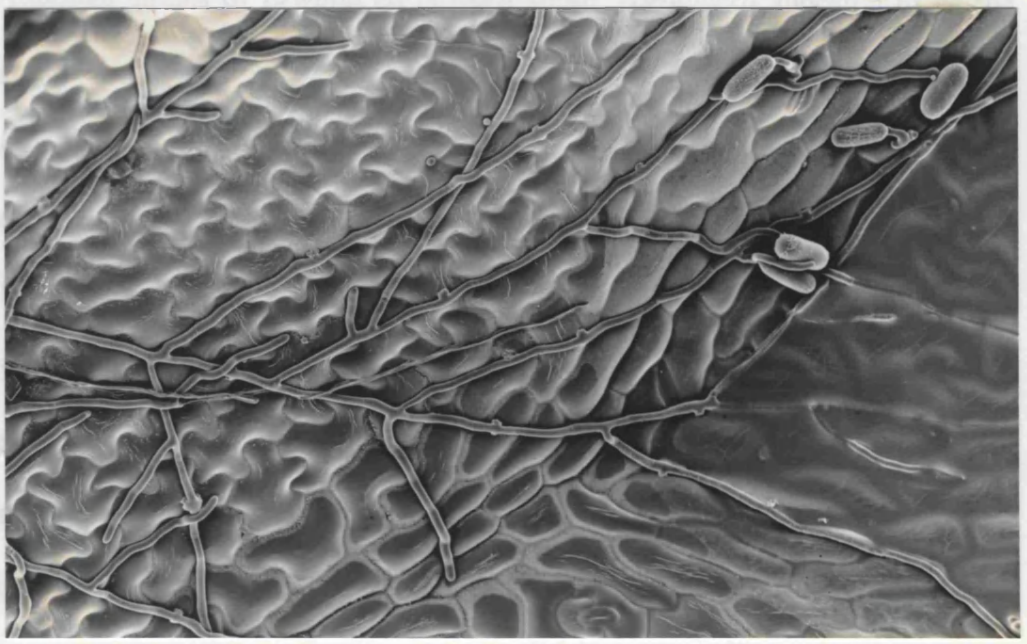


Plate 27: Extensive development of *E. heraclei* mycelium from conidia on cv. NRI-92, 15 hrs after inoculation. Note the paired appressoria arising from hyphae (×420)

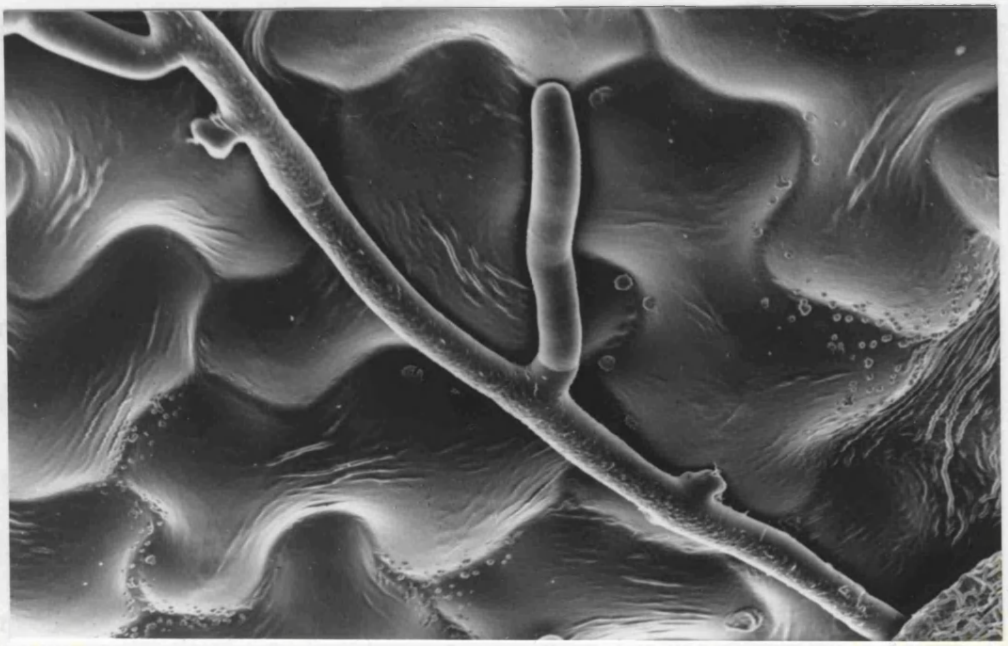


Plate 28: Detail of Plate 27 showing branching elongating secondary hypha with appressoria (×2080). Note residual ice crystals

6. DISCUSSION

Although strains of *Erwinia carotovora* subsp. *carotovora* appeared to be the most aggressive of the three soft rot erwinias (*Ecc, Eca, Ech*) tested in this study, certain differences in pathogenicity have also been observed within strains of *Erwinia carotovora* spp.

The aggressiveness of *Ecc* on carrot roots was in agreement with Michalik et al. (1992) who reported *Eca* strain SR 159 isolated from potato produced milder tissue rotting compared to *Ecc* strain SR 394 isolated from carrots. Host specificity is apparent among strains of *E. carotovora* for pathogenicity on carrot roots since it was shown that both bacteria can grow equally at 22 °C *in planta* (Pérombelon, 1982; Smith and Bartz, 1990). *Ecc* has a wide host range in both temperate and tropical areas while *Eca* is mainly associated with potato which is a cool climate crop (Lapwood et al., 1984; Pérombelon and Kelman, 1980). Although most soft rot erwinias are not host specific, certain differences in pathogenicity have been reported among strains of *E. carotovora.* All tobacco isolates of *Ecc* caused hollow stalk symptoms while non tobacco isolates of *Ecc* and isolates obtained from other plants caused necrosis on tobacco seedlings (Mc Intyre et al., 1978). The failure of certain *Ecc* strains to cause soft rot in different hosts was also observed by Smith and Bartz (1990). They showed that 2 out of 35 strains of *Ecc* were not pathogenic to potato stems incubated at 19 °C. Whitney and Mackey (1989) concluded that the environment and cultivar affected the aggressiveness of *E. carotovora* subsp. *betavasculorum* in sugar beet. This strain was highly pathogenic to plants grown in the greenhouse but was not so aggressive in the field. In the current study, for pathogenicity tests commercial taproots were obtained from both sources.

The minimum concentration of bacteria which gives reproducible infection under aerobic conditions should be employed to detect resistant somaclones and on this

basis 10^7 cfu/ml was chosen for assessment of pathogenicity. Inoculation of bacteria at densities of 10^7-10^8 cfu/ml at temperatures < 25 °C was also used by De Boer and Kelman (1978) for successful inoculation of potato tubers by *E. atroseptica* under aerobic conditions.

High concentrations of bacteria are generally used to obtain visible lesions on carrot roots under artificial conditions but such high population of bacteria would not be introduced into the plant in nature. Therefore, the strains of *Erwinia* used in this study that were either not pathogenic or were less aggressive on carrot tissues would be unlikely to initiate rotting on carrot under natural conditions. It was observed that pathogenic *Erwinia carotovora* produced larger lesions at 10^9 cfu/ml than at 10^7 cfu/ml. High numbers of non-pathogenic bacteria (such as some of the mutants cited above) may induce constitutive defence mechanisms after entering plant tissues which could further explain their inability to cause lesions (Lyon and Wood, 1976; Young, 1974); it was once considered that soft rotting bacteria such as *Ecc* are unusual amongst plant pathogens in not being able to elicit a hypersensitive-like reaction in plants (Kelman, 1979). However, recent findings have revealed that mutant of *E. chrysanthemi* EC16 was unable to secrete pectate lyase isozymes PelABCE$^-$ caused a typical hypersensitive response. Mutation of hypersensitivity response (HR) genes reduced the ability of *E. chrysanthemi* to induce hypersensitivity and to produce lesions in susceptible tobacco leaves (Bauer et al., 1995).

Soft rot bacteria such as *E. carotovora* are known to produce cell wall degrading enzymes. Several workers have demonstrated that the major pectic enzyme secreted by *Ecc* is PGL which is the main pathogenicity factor (Kotoujansky, 1987; Salmond, 1994). In addition, it has been suggested that endo-PG activity found in *Ecc* contributes to bacterial virulence (Willis et al., 1987).

Comparison of a range of mutants of *Ecc* differing in their PGL production revealed apparent differences in pathogenicity. The differences in pathogenicity and aggressiveness of mutants of *Ecc* from University of Warwick were related mainly to extracellular enzyme (PGL) production and secretion as indicated by other workers (Hinton and Salmond, 1987; Kotoujansky, 1987; Salmond, 1994). The secretory mutants are pleiotropically defective in the ability to excrete a variety of enzymes; they produced the same amount of PGL, endo-PG and cellulase as wild type but were impaired in their secretion of these enzymes into the extracellular environment and accumulated them in the periplasm (Hinton and Salmond, 1987; Murata et al., 1990; Palva et al., 1993; Pirhonen et al., 1991; Reeves et al., 1993). The extracellular enzyme deficient mutants, RJP 116 and RJP 243 were found to be non-pathogenic or less aggressive towards carrot roots and produced less PGL than wild type SCRI 193 *in planta* while the over producing extracellular enzyme mutant, PFP 16 caused greater rotting and there was higher PGL activity in infected roots than with the wild type at 10^9 cfu/ml.

Determination of growth *in planta* of mutants should lead to an understanding of which genotypic factors are required by a pathogen to multiply in host tissue and cause disease symptoms. All *Ecc* wild type and mutants obtained from University of Warwick increased in number with time in carrot roots. By contrast, the multiplication rate of wild type SCRI 193 was approximately twice that of enzyme deficient and hyper enzyme producing mutants. However, the rotting of carrot roots by wild type was not greater than the hyper enzyme producing mutant PFP 16. It can be concluded that symptom development on carrot roots was related to extracellular enzyme production and secretion rather than to bacterial populations in the carrot roots. PGL activity was not only detected in *Ecc* infected tissues but trace levels were found in healthy tissues. It

is possible that at least some of the activity was of host origin. Soroker et al. (1984) also found the activity of PL and PGL in crude extracts of healthy carrots. However, the existence of lyases in plant tissues has been questioned (Cooper, 1983).

The wild type and enzyme deficient mutants of *Ecc* from University of Missouri were found in this study to be either less aggressive or non-pathogenic on carrot taproots, a result in contradiction to that obtained by Murata et al. (1990) on potato tubers. This suggests that symptom production, following inoculation technique into carrot taproots represents a more rigorous assessment of pathogenic capability than does production of symptoms on potato tuber tissues.

In general, *Ecc* and *Eca* produce four PGL isoenzymes (PGLs ABCD) but only PGLs C and D of *Ecc* are excreted into the extracellular environment while the PGLs A and B are maintained in the periplasm of the pathogen (Hinton et al., 1989; Kotoujansky, 1987). The effect of isoenzymes on pathogenesis and their function in survival in various ecological habitats outside of host plants may be different (Salmond, 1994). Several defined PGL mutants of *E. chrysanthemi* and the wild type were less pathogenic on carrot roots in this study than *Ecc* obtained from University of Warwick. It would have been a more rigorous approach if they had been highly pathogenic to the carrot roots since we could have tested the virulence of mutants defective for only one PGL isoenzyme or for combinations of several PGL isoenzymes. Boccara et al. (1988) testing the virulence of several PGL mutants of *E. chrysanthemi* 3937 on *Saintpaulia ionantha* plants suggested that PGLs D, E and A were more important than PGLs B and C in virulence and systemic spread. They also showed that not all the different PGL isoenzymes were required to play a role sequentially to achieve an efficient degradation of the plant cell wall pectin because PGL BC double mutant was able to macerate *S. ionantha* plants. Collmer et al. (1982) revealed that degradation of pectic components

was not related to a nutritional requirement of *E. chrysanthemi* in the plant, because mutants incapable of growing on polygalacturonate but wild type for PGL secretion, retained their virulence and maceration capacity. The PGLs purified from *Ecc* have been tested on different plants to determine whether all isoenzymes were necessary for pathogenicity. Several workers have demonstrated that the alkaline isoenzymes of PGLs from *Ecc* play an essential role in maceration of potato tuber, carrot tissues or mitsumata (*Edgeworthia papyrifera* Sieb. et Zucc) bast (Lei et al., 1985a; Tanebe et al., 1987; Zink and Chatterjee, 1985).

Production of extracellular pectic enzymes of *Ecc* in the host-bacteria interaction does not necessarily implicate their involvement in pathogenesis in plant tissues, especially because they are also capable of eliciting phytoalexin synthesis (Palva et al., 1993; Yang et al., 1992). Davis et al. (1984) reported that in soybean, a PGL from *Ecc* appeared to play a role in the induction of plant defence mechanism. Culture filtrates from *Ecc* or pectinase containing preparations from other organisms elicited phytoalexin production, caused by release of oligogalacturonides from the plant cell walls. Yang et al. (1992) suggested that endo-PG released oligogalacturonides which were active as inducers of the host phenylalanine ammonia-lyase (PAL). No β-1,3-glucanase (pathogenesis related protein produced by plant tissues in response to bacteria) was induced in response to *Ecc* when exoenzyme negative mutants were inoculated into tobacco seedlings. These results indicate that pectic enzymes of *Ecc* possibly elicit the plant defence response by releasing pectic components from the plant cell wall that may function as endogenous elicitors (Palva et al., 1993).

The bacterial cell surface of *Ech* was also shown to play a significant role in virulence (Enard et al., 1988). However, there is no report about the cell surfaces of *Ecc* and *Eca.*

Variation in resistance to *Erysiphe heraclei* was apparent in the regenerated carrot plants and within the original seed line (NRI-92) without recourse to use of either chemicals or mutagens. Daub (1986) reported that the frequency of somaclonal variants selected at the plant level for a particular trait was estimated from 0.2% to 3%. In this study, 3% of the taproots regenerated from embryogenic suspension cultures derived from a susceptible cultivar of carrot was free from bacterial infection. However, 18% of the original seed line was also symptomless after inoculation with bacteria (Table 3.15). It was also noticed that the susceptible, regenerated taproots were more sensitive to *Ecc* than taproots from original seed line. These results indicate that taproots showing resistance to *Ecc* may have arisen either by variation induced by tissue culture or by the variation resulting from segregation in the seed line used.

In the studies of Sacristan (1982; 1986) with the rape *Phoma lingam* system, 22% of the regenerants from toxin selected cultures exhibited resistance or tolerance, while only 4% of regenerated plants from the control cultures become more tolerant.

The results of the present investigation suggest the possibility of obtaining plants resistant to *Ecc* from highly embryogenic cultures of carrot without applying any selection. This method has been used previously by many workers to select for disease resistant lines at the whole plant level (Larkin and Scowcroft, 1981; Sacristan, 1982; van den Bulk, 1991). Highly resistant celery plants to *Fusarium* have been obtained by screening somaclones without *in vitro* selection (Wright and Lacy, 1988). It is necessary to determine whether a resistance trait is heritable, and based on genetic not epigenetic changes; so selfed seed lines from regenerated taproots need to be tested. New resistant carrot lines would be very valuable for plant breeders and also could open a new experimental area for scientists to study the mechanism of resistance to *E. carotovora*. To date there is no evidence for the presence of resistance to *E. carotovora* on carrot,

therefore we were unable to use resistant cultivars of carrot to make any direct comparisons.

Because it is the most important species for causing cavity spot formation on carrot roots, investigations were carried out with *P. violae* (White, 1986; White et al., 1987; Vivoda et al., 1991). Higher incidence of cavity spot was obtained when the carrot roots were inoculated with V8 agar plugs infested with *P. violae*. Although the percentage of V8 agar plugs producing cavity spot on the carrot roots varied from 60-95%, generally 70-75% of the inoculation was successful. Similar results were demonstrated by White et al. (1987) and White (1988) using V8 agar plugs for inoculation of harvested carrot roots under laboratory conditions. Byrd (1988) reported that the incidence of the cavity spot lesions increased with the increase in age of carrot tissue. Vivoda et al (1991) also showed that the number of cavity spot lesions caused by *P. violae* and *P. ultimum* on 5-month-old carrots was as twice that on 3-or 4-month-old roots.

The higher susceptibility of the upper regions of carrot roots to cavity spot found here was in agreement with Davies et al. (1981) who reported that the lesions induced by *Mycocentrospora acerina* at the broken end of the taproots were more restricted than in the crown region. Blackish, rapidly spreading lesions caused by *M. acerina* were initially limited by suberized cells of xylem parenchyma, but there was little lignification and no wound cambium formation at the infected sites. Vivoda et al. (1991) also found that cavity spot lesions were concentrated in the upper parts of the carrot taproots although lesions existed over the entire roots.

Pythium spp. are known to penetrate most often directly through unwounded surfaces of their hosts by infection pegs or slender infection hype. Some can also enter through stomates and wounds (Chérif et al., 1991; Endo and Colt, 1974). Groom and

Perry (1985) found that wounding on carrot taproots did not enhance the development of cavity spot formation. In this study *P. violae* penetrated intact periderm directly. The resulting lesion could be especially damaging in the presence of soft rot erwinias because they do not enter unwounded periderm; *Ecc* produced extensive lesions only when co-inoculated with *P. violae* on intact carrot roots.

Evaluation of inoculum for *P. violae* clearly suggested that in general *P. violae* infested seeds provided a good source for determination of pathogenicity. Barley seeds appeared to be the most suitable for growth of *P. violae* and produced the larger cavities on carrot roots. However, using V8 plugs as the inoculum source gave a similar frequency of cavity spot lesions. Also because growth of *P. violae* on barley seeds was longer than on V8 agar, V8 plugs were used for routine inoculation. Vivoda et al. (1991) reported that typical cavity spot lesions occurred on carrot taproots grown in potting mix artificially infested with *P. violae* while superficial and discoloured lesions occurred on the harvested carrot roots inoculated with corn meal agar plugs at the laboratory conditions. Therefore, it is also necessary to determine whether laboratory screening gives positive correlation with direct field assessment tests (White et al., 1987).

It was evident from this investigation that *P. violae* is capable of producing cell wall degrading enzymes *in vitro* and *in vivo*. Extensive growth of *P. violae* occurred when glucose was the sole carbon source in the media, which is similar to results found by Schrandt et al. (1994) with *P. violae* and Janardhanan and Husain (1974) with *P. butleri* and Winstead and McCombs (1961) with *P. aphanidermatum.* When pectin, cellulose and carrot cell walls were used as carbon sources, growth of *P. violae* was extremely poor as reported for *P. butleri* on similar substrates (Janardhanan and Husain, 1974).

Although the production of cell wall degrading enzymes by *P. violae* has not been studied in detail, that of other *Pythium* spp. has been examined. Winstead and McCombs (1961) showed that *P. aphanidermatum*, the cottony-leak disease of cucumber was able to produce cellulases *in vitro* and *in vivo*. Later studies confirmed that other, but not all, *Pythium* spp. were capable of producing cellulases. No cellulase activity was detected in seven-day-old culture filtrates of *P. irregulare, P. perniciosum* and *P. dissotocum* while *P. sylvaticum* produced cellulase (Nemec, 1974).

Cellulase was the main enzyme produced *in vitro* by *P. violae* in this study. Production of cellulase by this pathogen occurred on all carbon sources used. This is in agreement with the finding of Sadik et al. (1983) who studied *P. aphanidermatum,* stalk rot of maize. They showed that least amount of cellulases was produced in culture filtrates of *P. aphanidermatum* without addition of glucose as a starter while activity was greater in the medium containing cellulose and glucose.

In general, fungi produce cellulases *in vitro* more abundantly when the only available carbon source in the medium is cellulose (Wood, 1967). This was the case with *P. violae* suggesting that cellulase is being induced; the detection of cellulase in this study when pectin was used as a carbon source presumably represents the basal synthesis of cellulase by *P. violae*. *P. violae* produced the least amount of cellulase on medium containing carrot cell walls. By contrast, in *Verticillium albo-atrum* and *Fusarium oxysporum*, tomato cell walls stimulated high production of pectinases, arabinases, xylanases and cellulases (Cooper and Wood, 1975). Although cell walls are insoluble, and cannot directly induce the synthesis of cell wall degrading enzymes, they provide inducers after degradation by small amounts of basal enzyme produced constitutively by pathogens as reported by Cooper and Wood (1975). This poor ability of *P. violae* to degrade insoluble polymers may reflect its limited saprotrophic ability.

The production of cellulase by *P. violae* was also detected *in vivo* and was found late in lesion formation. This correlates with general evidence to suggest that cellulases are produced late in the infection process in many diseases e.g. in pink rot of onion (*Pyrenochaeta terrestris*) first pectic enzymes were produced while cellulases were detected during later stages of rotting (Cooper, 1984).

Although there was no attempt to define which type of cellulase was produced, it was found that cellulase was extracellular in nature and did not remain bound to the fungal hyphae.

Evidence from studies on other *Pythium* spp. like *P. aphanidermatum, P. ultimum* and *P. butleri* shows that polygalacturonase is produced by *Pythium* spp. (Janardhanan and Husain, 1974; Mellano et al., 1970; Winstead and McCombs, 1961). However, no evidence of this enzyme from *P. violae* was found either *in vitro* or *in vivo.*

Other pathogens of carrot such as *Mycocentrospora acerina* secrete pectinases to hydrolyse the rhamnogalacturonan present in the middle lamella; it was discovered that the aggressiveness of isolates directly correlated with the activity of pectinolytic enzymes from the pathogen (Davies and Lewis, 1981).

PL activity from *P. violae* was discovered in response to pectin substrates and, unusually, to cellulose substrates although results were erratic. Activity was higher in the high salt buffer washes from mycelia that had been grown on pectin suggesting that this enzyme remains mostly bound to the mycelia.

Suberin is similar to cutin in that both contain aliphatic monomers held together largely by ester linkages (Kolattukudy, 1980), therefore suberinase degrading enzyme activity can be studied by the assay methods developed for cutinase. Although *P. violae* penetrates intact taproots of carrot *via* the suberinized periderm, it grew very poorly on media containing suberin as the sole carbon source; however *P. violae* produced an

extracellular esterase, which may be suberinase, when grown in a medium supplemented with potato suberin. Esterase levels were not affected by sucrose used as a 'starter' in the medium. Only a few fungi are able to penetrate thickened cell walls of periderm by degradation of suberin. For example; *Gaeumannomyces graminis* on wheat, *Colletotrichum gloeosporioides* on papaya fruit, *Armillaria mellea* on forest trees and *Streptomyces scabies* on potato (Cooper, 1983; Kolattukudy and Crawford, 1987; Zimmermann and Seemüller, 1984). Fernando et al. (1984) reported that extracellular fluids from the suberin grown *Fusarium solani pisi* catalysed the hydrolysis of *p*-nitrophenyl butyrate and radioactive apple cutin. Isolation, purification and characterisation of fluids showed that suberinase and cutinase were similar in their catalytic properties and amino acid composition. Zimmermann and Seemüller (1984) reported suberinase activity in suberin grown culture fluids of *Fusarium solani* f. sp. *pisi* and *Armillaria mellea.* Fernando and co-workers (1984) showed that suberinase of *Fusarium solani pisi* isolate T-8 binds very tightly to cells and/or suberin in the medium. More than 90% of the polyesterase activity could be recovered by washing the suberin containing mycelial mat with 0.4% Triton X-100 in 0.2 M Sodium phosphate buffer. This procedure yielded 13 times as much esterase activity as that contained in the extracellular fluid. *P. violae* may be similar in this respect but extraction was not attempted here. Electron microscopy studies of *Hevea brasiliensis* infected with both *Rigidoporus lignosus* and *Phellinus noxius* also revealed that haustorium-like structure of fungi penetrated suberized cell walls of rubber tree roots by a combination of mechanical and enzymatic action (Nicole et al., 1986).

Despite the direct evidence gained that *P. violae* does produce cell wall degrading enzymes this study does not prove their role in pathogenesis. It is necessary to determine whether these enzymes are pathogenicity determinants or if they contribute to

virulence. However, the range of enzymes produced *in vitro* and *in vivo* appeared superficially similar. Symptoms of the water soaked lesions observed by two days appeared to be correlated with the activities of the pectinolytic and cellulolytic enzymes produced. Future work should involve transmission electron microscopy (TEM) to reveal the nature of damage to carrot root cells and cell walls. Genetic studies with defined mutants will not be easy with a slow growing organism which has not previously been genetically manipulated.

Screening somaclones for resistance to *P. violae* showed that 6% of the regenerated individual taproots were uninfected with *P. violae*., and also the proportion of inoculated sites infected with *P. violae* was less than in the original seed line. But, both of them were similar in terms of mean diameter of cavities. As mentioned before, the high percentage of resistant individual roots does not coincide with Daub's (1986) suggested frequency range of a trait arising from somaclonal variation.

The results of the present investigation suggest that there is the possibility of obtaining plants resistant to *E. heraclei via* callus cultures of carrot hypocotyl explants. Screening unselected carrot regenerants at the whole plant level for resistance against powdery mildew resulted in 27% of regenerated carrot plants produced from callus culture as disease resistant. However, this finding is obviously far higher than the expected extent of variation resulting from somaclonal variation (Daub, 1986; Larkin and Scowcroft, 1981). The percentage of symptomless plants (*ca.* 40% see Section 4.1) in the original seed line was also found to be very high. These results suggest that the high frequency of powdery mildew resistance was likely due to segregation expressed in a seedling population from germplasm with an initially high level or frequency of powdery mildew resistance (Daub, 1986). Nevertheless, McCoy et al. (1982) showed that the genotype of the plant can affect the amount of variability obtained from tissue

culture. Wright and Lacy (1988) obtained high frequency of resistant celery plants to *Fusarium* by using moderately resistant cultivars rather than a population derived from highly susceptible material. Sebastiani et al. (1994) also screened potato plants from callus cultures of potato cv. Désirée which was tolerant to *Verticillium dahliae*. Dry weight, leaf area and tuber production of both control cv. Kondor (resistant to *V. dahliae)* and resistant clone R3-90 decreased after inoculation with *V. dahliae*. These results confirmed that the selected clone was similar to the resistant cultivar.

Eventually it was shown in this study that regenerated plants were more resistant to powdery mildew than the original seed line. When the experiment was initiated there were no references concerning resistance of carrot to these three pathogens (*E. heraclei, E. carotovora, P. violae*). Susceptibility of regenerants would depend upon the seedling from which they were derived i.e. the chance for selection of disease resistant clones would increase dramatically if by chance they had originated from a resistant seedling. In conclusion, resistant Somaclone-4 was most likely derived from variation in the original seed line (NRI-92) which was resistant to powdery mildew. Resistance to powdery mildew in regenerated plants was stable and that resistant line could be incorporated into a breeding programme.

Initial work using scanning electron microscopy revealed no obvious differences between germination of conidia, or appressorium formation on the susceptible original seed line NRI-92 and on the regenerated Somaclone-4 plants. In this study, *E. heraclei* had cylindrical conidia and unforked germ tubes as described by Jenkins et al. (1985). However, the frequency of elongating secondary hypha reflecting haustorium formation in a compatible host, was higher on NRI-92 compared to Somaclone-4. Similar results were also obtained by Cohen et al. (1990) who did not find differences between resistant and susceptible muskmelon cultivars to powdery mildew caused by *Sphaerotheca*

fuliginea up to and including the stage of haustorial initial formation. Callose-like material in the penetration zone of both susceptible and resistant leaves was observed. But, most haustoria in resistant cultivars did not produce lobes and this coincided with collapsing epidermal cells and resulted in death of the attacking germlings.

Both preinfectional resistance and postinfectional resistance have been implication in resistance to *Erysiphe* spp. Preinfectional resistance may be expressed in the form of reduced germination or appressorium formation and postinfectional resistance can be associated with papilla formation or hypersensitive death of epidermal plant cells (Kogo et al., 1988; Cohen and Eyal, 1988). Some structural characteristics include the amount and quality of wax and cuticle that cover the epidermal cells, water-soluble inhibitors on the surface of the plants, the structure of the epidermal cell walls, the size of pre-existing features in plants which can act as physical barriers and block pathogens entry into plant tissues. Thus, pre-existing surface structures or inhibitors can not be the cause for resistance to *E. heraclei*. This suggests that in the resistant line infection may be stopped following penetration of the leaf.

The development of *Erysiphe* spp. on host and non host plants was studied by Staub et al. (1974). Light- and scanning electron microscopy revealed that resistance was expressed to *E. cichoracearum* on non-host barley leaves before penetration occurred; germination occurred successfully, but germ tubes were not capable of dissolving the epicuticular structures, thus, it failed to penetrate epidermal cells. However, in the case of *E. graminis* on non-host cucumber plants, resistance was expressed after penetration of the epidermal cells. *E. graminis* penetrated the epidermal cells but its haustorial development was stopped by a hypersensitive reaction before branching of the haustorial initials. They suggested that dissolution of wax crystals on barley might be critical for direct penetration by this fungus. Ultrastructural microscopic

studies of susceptible muskmelon (*Cucumis melo*) to *Sphaerotheca fuliginea* by Cohen et al. (1990) revealed that the first penetration occurred from a single-celled primary germ tube at 20-24 hrs after inoculation, then second and third penetrations were followed by a second germ tube which appeared at 36-48 hrs. However, in resistant interactions the fungus mostly produced a single germ tube which resulted in a single penetration. Later, in the penetrated epidermal cells a swollen haustorium was heavily encased with callose-like material. Kunoh et al. (1985) showed that cytoplasmic aggregates in barley coleoptile cells were induced by *E. pisi* appressoria, a non-pathogen of barley, just before penetration.

Also induction of fluorescent antifungal compounds such as lignin and callose in response to infection has been observed in a number of species as an indication of resistance (Southerton and Deverall, 1990; Tiburzy and Reisener, 1990). Cohen and co-workers (1990) also showed that in cultivars resistant to *Sphaerotheca fuliginea*, accumulation of phenolic compounds, callose deposition, lignification and necrosis of the fungus and epidermal cytoplasm occurred after initial penetration at about 24 hrs after inoculation. Kogo et al. (1988) showed that autofluorescent HR of epidermal cells of barley with a gene for resistance to *E. graminis* f. sp. *hordei* first occurred in 72% of attacked epidermal cells at 24 hrs. Cohen and Eyal (1988) also reported that reduced fungal growth in resistant genotypes of *Cucumis melo* at 16-24 hrs after inoculation with powdery mildew *Sphaerotheca fuliginea* was associated with a hypersensitive reaction.

Suppression of host defences must occur in compatible host-parasite combinations. This may partly explain in susceptible varieties of barley, that inhibition of phenylalanine ammonia lyase (PAL) and cinnamyl alcohol dehydrogenase were found to be associated with *Erysiphe graminis* infection (Carver et al., 1994).

CHAPTER 3

RESULTS AND DISCUSSION

III - SELECTION OF PLANT CELLS FOR DISEASE RESISTANCE

1. INTRODUCTION

Co-cultures of either bacteria or fungi and plant cell suspension cultures can be useful for examining plant-pathogen interactions and for selection of either resistant or tolerant cells ultimately to improve the disease resistance of plants.

Successful selection using embryogenic suspension cultures and either the pathogen itself or its products e.g. toxins, as the selection agent, has been reported to give increased disease resistance for several plant species (van den Bulk, 1991).

The use of fine embryogenic suspension culture and the pathogen as the selection agent for selection of disease resistance has obvious advantages for several reasons including:

1. Small uniform cell aggregates and embryos are bathed by the culture medium which maximises contact of pathogen and pathogen products with all host cells;
2. Strict control of ambient conditions e.g. temperature;
3. Large numbers of cells can be challenged by pathogen;

4. If successful, the system could be used for testing the possible involvement in pathogenicity bacterial toxins or enzymes.

Therefore, it was decided to use a highly embryogenic cell suspension cultures of carrot for selection for resistance to both *Erwinia carotovora* and *Pythium violae*.

2. CO-CULTURES OF PLANT AND BACTERIAL CELLS

2.1. DETERMINATION OF OPTIMUM MEDIUM FOR PLANT AND BACTERIAL CELLS

The aim of these experiments was to find a medium in which carrot cells and bacterial cells would survive at high levels in control treatments and bacterial cells would not multiply. If the medium was beneficial only to plant cells or to bacterial cells, a true reflection of the interaction may not be presented.

2.1.1. Survival of Carrot Cells in Different Media

Ten-day-old carrot cells were prepared as in Materials and Methods (Chapter 2), Section 1.4.4. Five ml suspension containing 2.5 ml PCV was then added to 45 ml of the following media in 250 ml screwtop conical flasks. The tested media were as follows :

1/2 MS pH 5.7

1/10 MS pH 5.7

Milli Q pH 6.0

Culture A pH 6.0 (175 mM mannitol, 0.5 mM MES, 0.5 mM $CaCl_2.2\ H_2O$, 0.5 mM K_2SO_4) (Dr. D. Dymock, pers. comm.)

MS2 + 5 μM 2,4-D pH 5.7

Two replicated flasks were set up for each treatment and were incubated as explained in Materials and Methods, Section 1.4.4.

All the media used maintained viability of the cell units and single cells up to 4 days (Figure 3.6). The survival of carrot cells decreased slightly i.e. 1.2-23% for cell units and 5.5-18.9% for single cells in Milli Q (MQ), 1/2 MS and 1/10 MS media within 6 days (Appendix 22a, 22b).

Culture A did not result in reduced viability even after 6 days. The cells suspended in basal MS medium supplemented with 2% sucrose and 5 μM 2,4-D showed increased viability in 6 days.

2.1.2. Survival of Bacterial Cells

Ecc and *Eca* were prepared as described in Materials and Methods, Section 2.1. The number of bacteria were determined spectrophotometrically and adjusted to 10^8 cfu/ml. Five ml from this suspension was spun down (10 min, 4000×g), resuspended in a fresh medium and then added to 45 ml of the following media in screwtop flasks to give a final concentration of 10^7 cfu/ml: 1/2 MS (pH 5.7), 1/10 th MS (pH 5.7), MQ (pH 6.0), Culture A (pH 6.0) and MS2 supplemented with 5 μM 2,4-D (pH 5.7).

These flasks were incubated at 25 ± 1 °C in a shaker at 100 rpm under light (30 μmol m^{-2} s^{-1}). Each treatment had 3 replicated conical flasks. Bacterial growth was recorded at daily intervals by serially diluting 0.1 ml aliquots from each flask and spreading 0.1 ml of the appropriate dilutions onto three NA plates. Inoculated plates were kept at 30 ± 2 °C for 2 days then the number of bacterial cells present on each plate was counted.

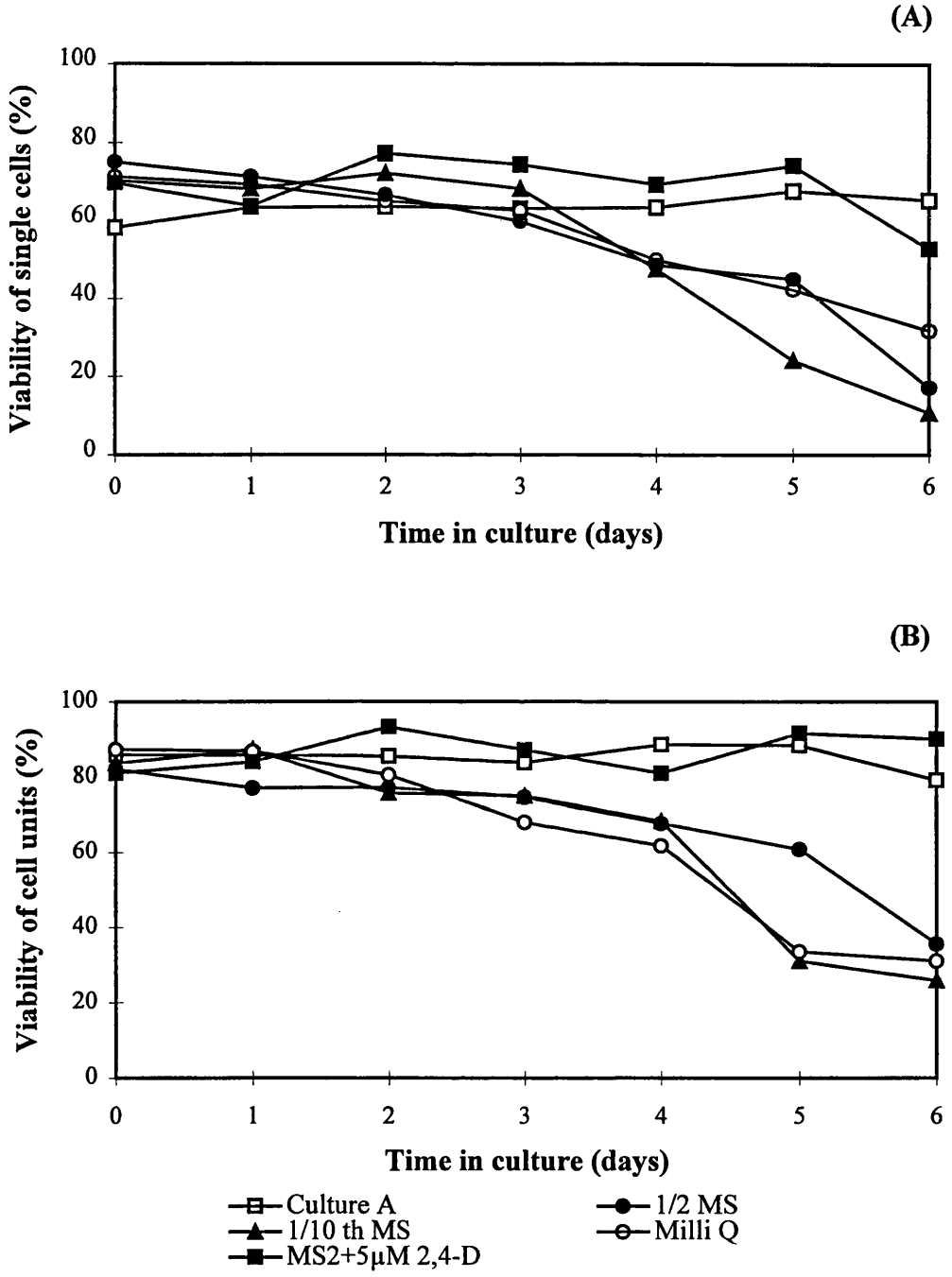


Figure 3.6: The mean percentage viability of suspension cultured single cells (A) and cell units (B) of carrot cv. Morot Duke

The treatments had significant ($p < 0.01$, Appendix 22a, 22b) effects on the survival of carrot single cells and cell units as did the length of time and the interaction between time and treatment. Culture A and MS2 + 5 μM 2,4-D contributed similarly to the viability of plant cells according to Duncan's Multiple Range Test at 5% level after 6 days in culture.

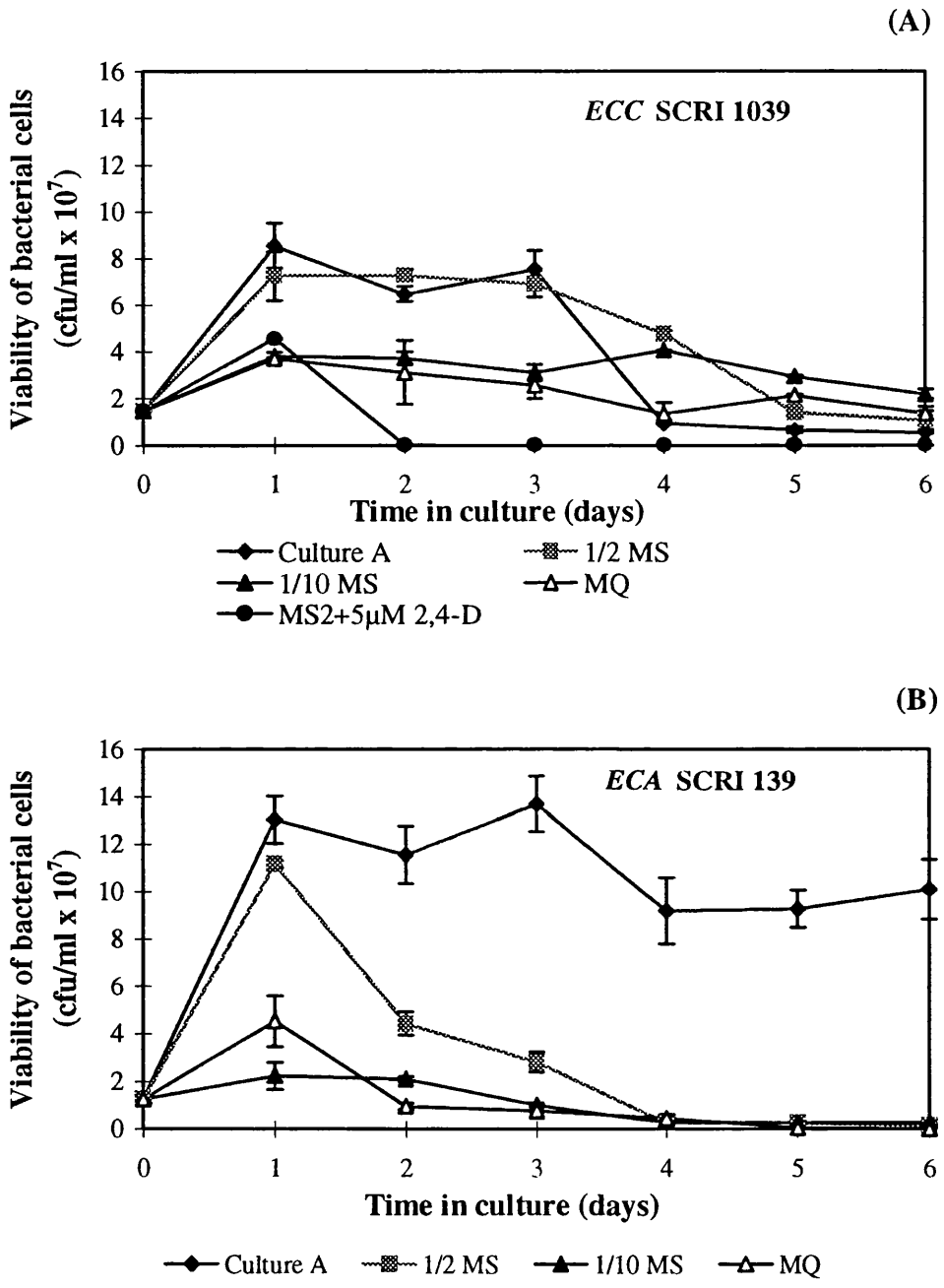


Figure 3.7: The survival of bacterial strains (A) *Ecc* SCRI 1039 (B) *Eca* SCRI 139 suspended in various media

The mean of 3 replicated readings for each treatment was plotted with standard deviation. The media, time and the interaction between time and media had different effects on the bacterial population ($p < 0.01$). The Duncan's Multiple Range Test at 5% level demonstrated that the increase in MQ and 1/10 th MS media for *Ecc* and *Eca* was less compared to Culture A and 1/2 MS media in 1 day while there were no bacteria after 3 days in the original medium (MS2 + 5 µM 2,4-D).

The results are shown in Figure 3.7. The population of *Eca* and *Ecc* increased *ca.* 10 and 5 fold respectively in both 1/2 MS and Culture A in 1 day ($p < 0.01$, Appendix 23a, 23b). There were no bacteria after 3 days in the original medium (MS2 + 5 μM 2,4-D).

Therefore, either MQ or 1/10 strength MS could be a suitable medium for future co-culture experiments since both media maintained high survival of plant cells up to 5 days and bacterial number did not increase as much as in the other media. Furthermore, as these media do not contain a carbon source, the probability of host cell death occurring as a result of accumulation of spurious toxic metabolites from extensive bacterial growth is reduced. A single medium, 1/10 th MS, was chosen for the co-culture experiments. Since 1/10 th MS medium was diluted MS basal medium, the adaptation of carrot cells in co-culture experiments should be easier than with MQ.

2.2. THE EFFECT OF AGE OF CARROT CELLS ON CELL KILLING

Carrot cells from cultivar Morot Duke in lag (2-day-old), in log (8-day-old) and beginning of the stationary (14-day-old) phases were studied to determine the influence of age on response to *Ecc*.

Carrot cells and *Ecc* cells were prepared as described in Materials and Methods, Section 1.4.4 and 2.1 respectively. The plant cells were suspended at 1.0 ml PCV (after centrifugation for 2-3 min at 380×g) in 100 ml conical flasks containing 20 ml of 1/10 th MS. 2.22 ml of the bacterial suspension (SCRI 1039) with 10^8 cfu/ml was added to this media to give a final concentration of 10^7 cfu/ml. Plant cells without bacteria were used as control. There were 2 replicated flasks for each treatment. The flasks were incubated as described in Chapter 2, Section 1.4.4.

Co-culture of carrot cells with SCRI 1039 resulted in death of plant cells, although the susceptibility of single cells and cell units varied with the age of host cells. Time and treatments had a highly significant effect on viability of cell units and single cells from 2, 8 and 14-day-old suspension cultured cells of carrot inoculated with *Ecc* ($p < 0.01$, Appendix 24a, b, c, d, e and f). The viability of treated cell units decreased with time significantly in 8 and 14-day-old suspension cultured cells whereas the viability of single cells for all treatments and 2-day-old cell units were similar up to 1.5 hrs, after which it decreased rapidly. Figure 3.8a and b show that none of the 2-day and 14-day-old cultured single cells and cell units survived in 1/10 strength MS media more than 1 day while 8-day-old culture remained viable up to 2 days. SCRI 1039 caused 100% mortality by 2 days in 2 and 14-day suspension cultured cells while 17% cell units and 6% single cells were remained alive in 8-day-old suspension culture. Also, the viability of the untreated controls of single cells taken from 2-day-old and 14-day-old cultures decreased rapidly by 1 day while there were viable single cells in the cultures of 8-day-old suspensions.

Therefore, log phase (8-day) was selected as the most suitable age for future co-culture experiments because a high percentage of plant cells survived in 1/10 th MS medium control beyond 2 days and a proportion remained alive after 2 days following co-culture with *Ecc*; these survivors may provide suitable challenged material for regeneration.

2.3. KINETICS OF CELL KILLING

Previous results had suggested that *E. carotovora* killed carrot cells very rapidly. Hence it is the purpose of these experiments to examine closely the early kinetics of cell killing by *E. carotovora* in order to determine when the actual cell killing was started.

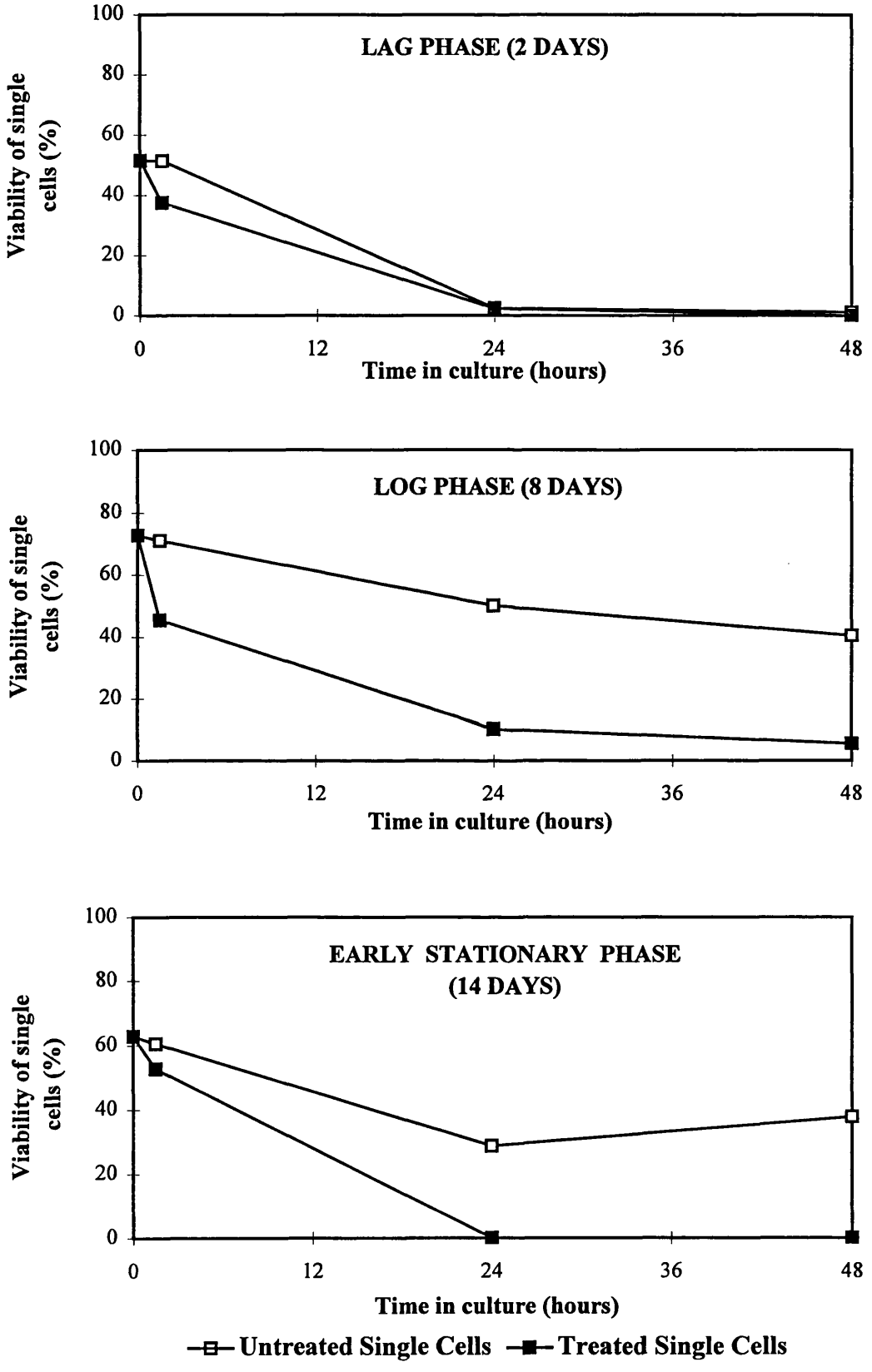


Figure 3.8a: Viability of single cells from 2, 8 and 14 day old suspension cultured cells of cv. Morot Duke inoculated with *Ecc* SCRI 193

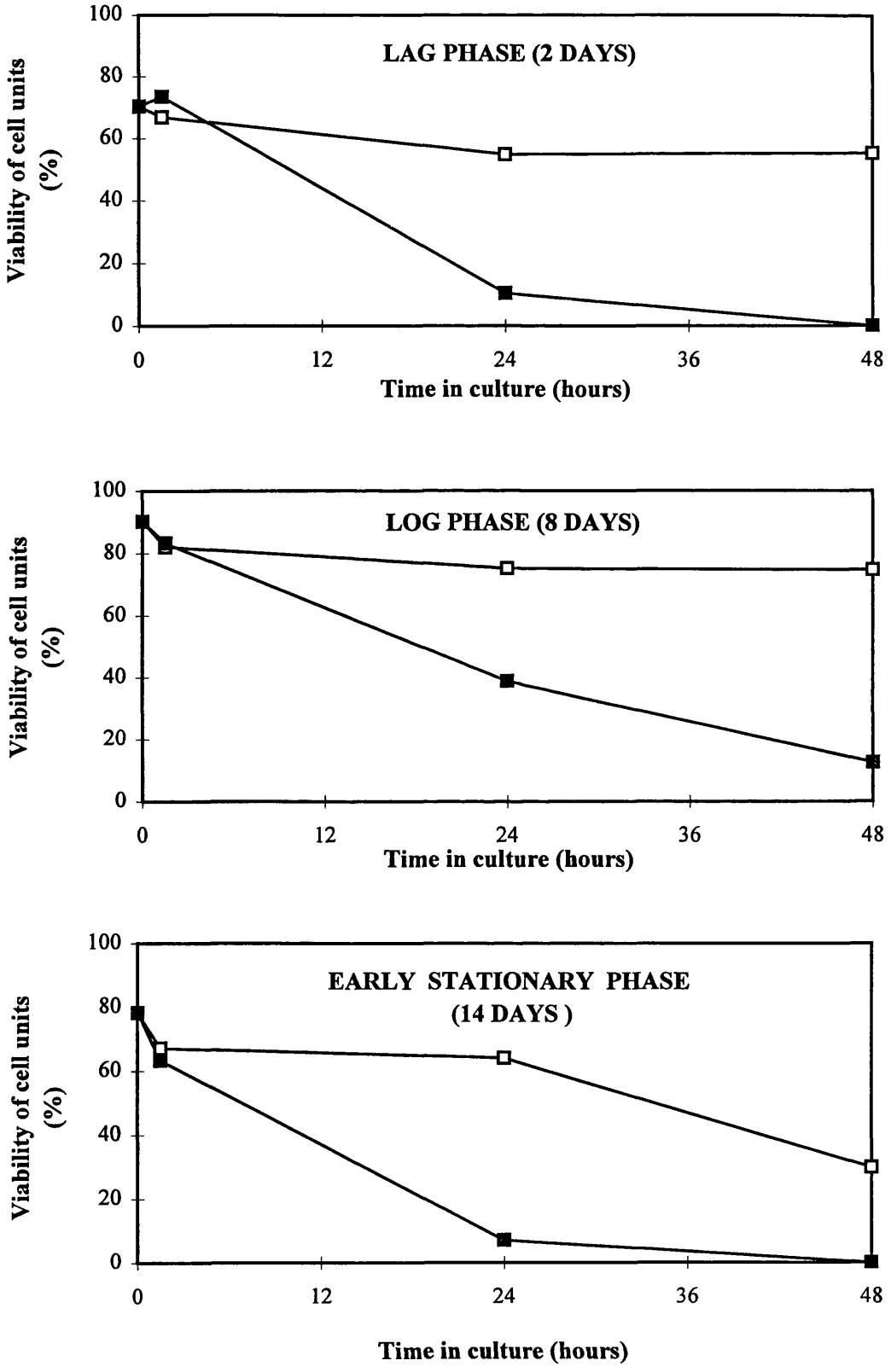


Figure 3.8b: Viability of cell units from 2, 8 and 14 day old suspension cultured cells of carrot cv. Morot Duke inoculated with *Ecc* SCRI 193

Ten-day-old carrot suspension culture was prepared as described in Materials and Methods, Section 1.4.4 and inoculated into 250 ml conical flasks with 45 ml 1/10 th MS at 2.5 ml PCV after centrifugation for 2-3 min. The bacteria (SCRI 1039) were grown overnight (13 hrs) in 30 ml sterile universal with 30 ml LB broth. To reveal the effect of different inoculum concentrations on the killing of carrot cells, a 10-fold dilution series (10^9 to 10^7 cfu/ml) of bacterial suspension was prepared with sterile distilled water. Two conical flasks were set up for each treatment. Plant cells untreated with bacteria were used as control. Conical flasks were maintained as described in Materials and Methods, Section 1.4.4.

Co-culture with bacteria resulted in death of carrot single cells and cell units almost within 2 days (not shown in Figure 3.9 for clarity). 10^7 cfu/ml caused 81% and 60% cell mortality on single cells and cell units respectively in 14.5 hrs while 10^8 and 10^9 cfu/ml caused 64% and 23% cell mortality on cell units during the same time respectively. There was a positive and significant correlation between time and the killing of carrot cells ($p < 0.01$, Appendix 25a, 25b). The killing of carrot cells increased with time for all inoculum concentrations used. Significant killing of the carrot cell units in the inoculated treatments occurred within 1.5 hrs following inoculation (Figure 3.9). However, after 14.5 hrs the speed of cell killing had greatly increased at 10^7 and 10^8 cfu/ml, while with 10^9 cfu/ml it was less effective by that time. In contrast, single cells were less sensitive to 10^8 cfu/ml compared to 10^7 and 10^9 cfu/ml (Duncan's Multiple Range Test at 5% level). Thus, it would appear that carrot cell killing first occurs between 5 and 15 hrs following contact with *Ecc.*

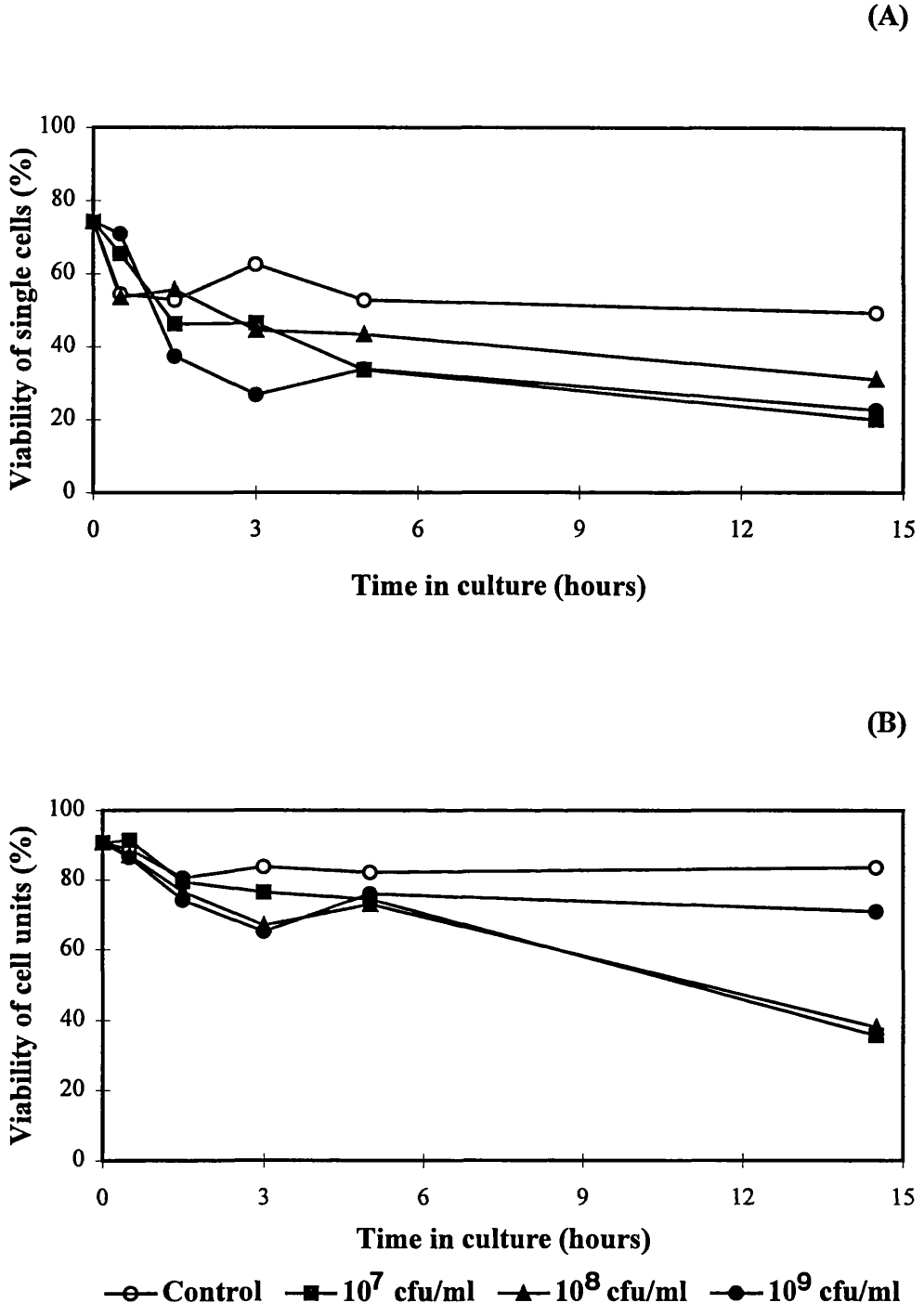


Figure 3.9: Killing of carrot cv. Morot Duke embryogenic suspension cells by *Ecc* (A) single cells, (B) cell units

The killing of carrot cells changed significantly ($p < 0.01$) with the inoculum concentrations used. The interaction between time and concentrations was also significant ($p < 0.01$).

2.4. THE EFFECT OF NUMBER OF BACTERIA ON CELL KILLING

The object of this experiment was to find the effect of the bacterial numbers on cell killing.

Ecc (SCRI 1039) was grown in 100 ml conical flasks containing 30 ml LB broth. The number of bacteria was determined spectrophotometrically and adjusted to 10^8 cfu/ml. Ten-day-old carrot cells were inoculated into 250 conical flasks containing 45 ml of 1/10 th MS to obtain a final density of 2.5 ml PCV. Five ml of the bacterial suspension was spun down (10 min, 4000×g) and resuspended in a fresh medium. 10^8, 10^6 and 10^4 cfu/ml bacteria were added to the above media to give final concentrations of 10^7, 10^5 and 10^3 cfu/ml. Uninoculated plant cells were set up as controls. There were 2 replicates from each treatment. The conical flasks were incubated as explained in Materials and Methods, Section 1.4.4.

Exposure to SCRI 1039 reduced the survival of single cells and cell units of the carrot suspension culture. The effect was significantly proportional to inoculum concentration. Thus, after 7 hrs, cell killing (below control values) by 10^3, 10^5 and 10^7 cfu/ml was 46, 41 and 19% for single cells and 80, 67 and 47% for cell units respectively (Figure 3.10).

Figure 3.10 shows that the majority of plant cells were killed by bacteria within 3 days. The mortality of single cells ranged from 98% at 10^3 cfu/ml to 100% at 10^7 cfu/ml within 2 days, while the mortality of cell units ranged from 26% at 10^3 cfu/ml to 88.4% at 10^7 cfu/ml within same period. Killing of single cells by SCRI 1039 was faster than that of cell units.

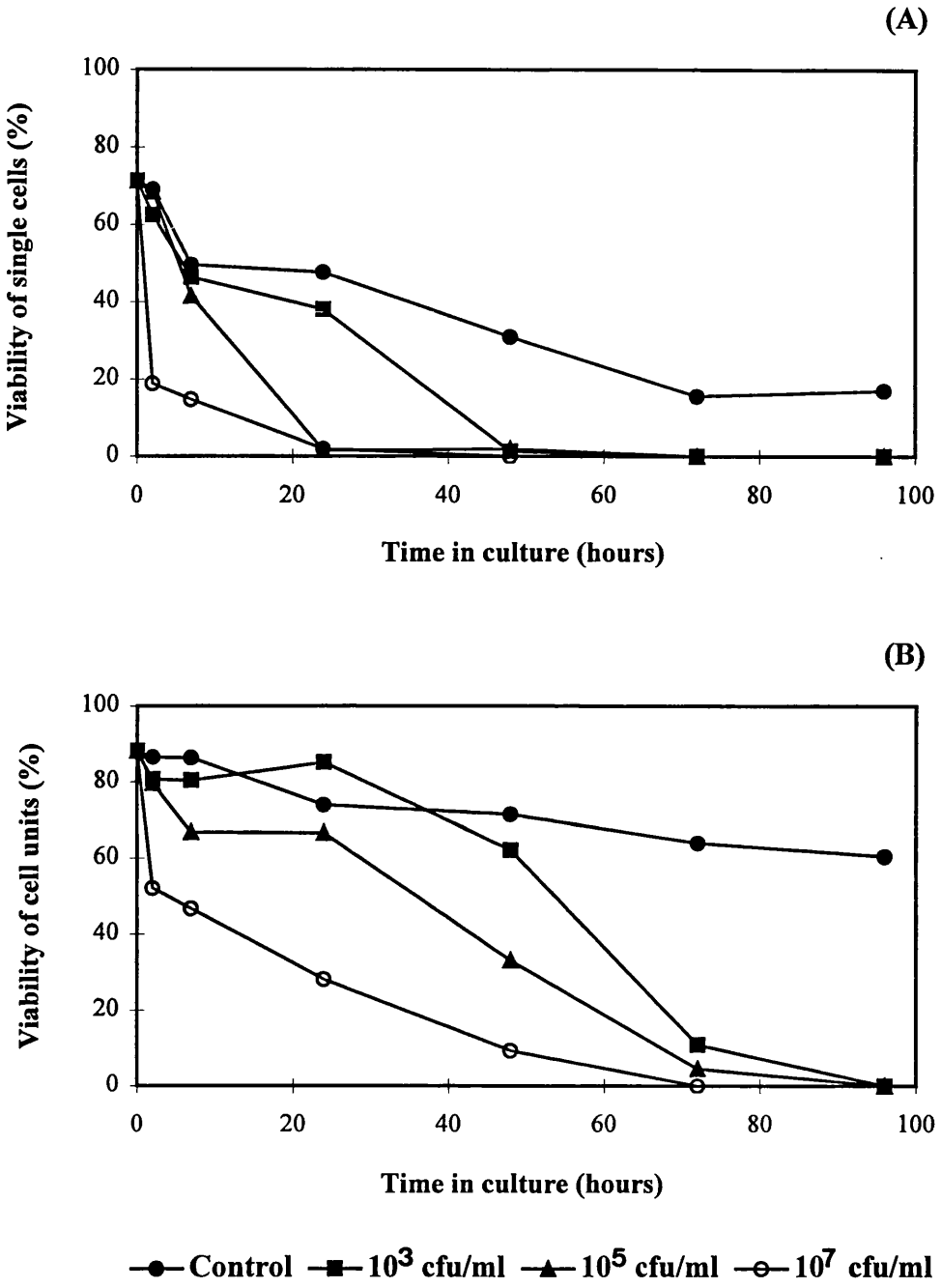


Figure 3.10: Determination of aggressiveness of *Ecc* on viability of single cells (A) and cell units (B) at 10^3, 10^5 and 10^7 cfu/ml

The number of bacteria had a significant effect ($p < 0.01$) on the viability of single cells and cell units as did the length of time and interaction between time and different bacterial numbers (Appendix 26a, 26b). 10^7 cfu/ml bacterial inoculum resulted in much more severe killing of both single cells and cell units than the other concentrations used according to Duncan's Multiple Range Test at 5%.

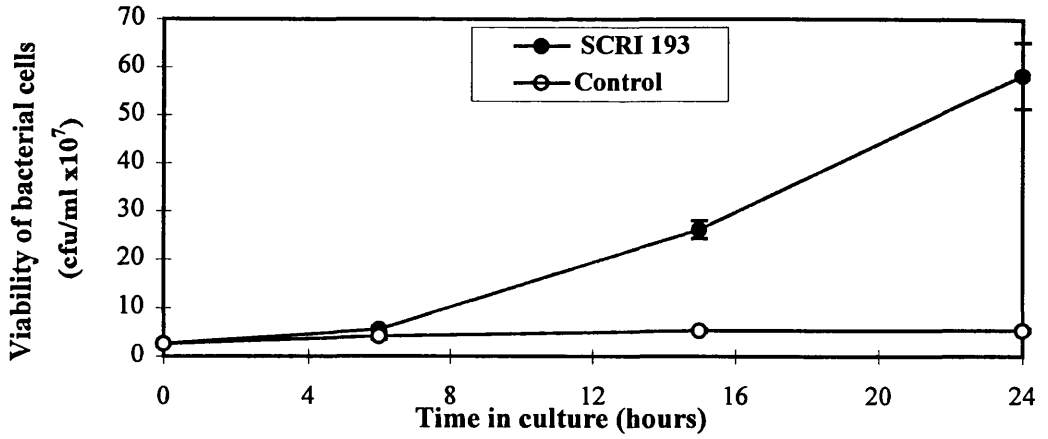
2.5. GROWTH OF BACTERIA IN CO-CULTURE

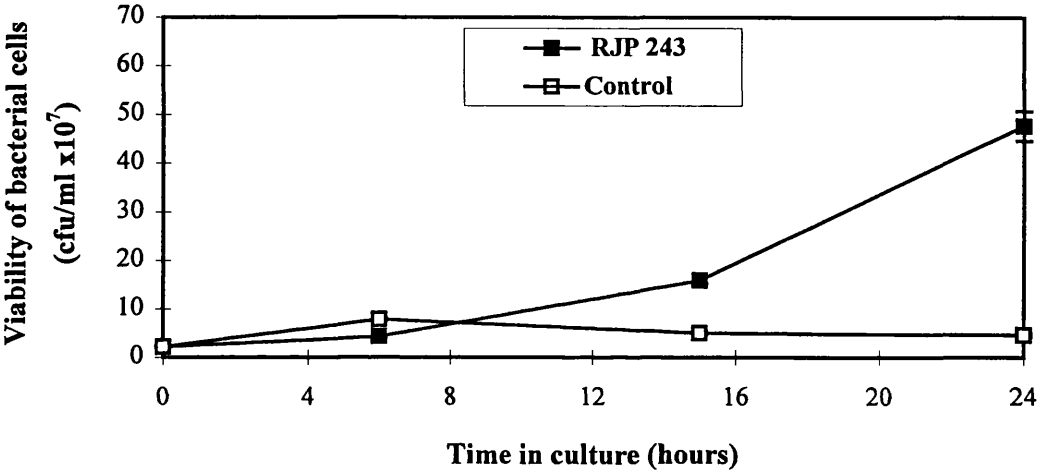
The objective of a subsequent experiment was to detect if the production of pectate lyase was related to growth and multiplication of *E. carotovora* in carrot embryogenic suspension cells. To do this, growth of wild type and pectate lyase deficient mutants were compared.

Twenty ml 1/10 th MS medium containing carrot embryogenic cells at 1 ml PCV (after centrifugation at 380×g for 2-3 min) was placed into 100 ml conical flasks and 2.2 ml, 10^8 cfu/ml bacterial suspensions was inoculated into plant cells to obtain a final concentration of 10^7 cfu/ml. Three replicated flasks were set up for each bacterial strain and incubated as explained in Materials and Methods, Section 1.4.4. Flasks containing bacteria only in 1/10 th MS media were used as controls. Bacterial number was evaluated at 0, 6, 15 and 24 hrs.

Figure 3.11 illustrates that all isolates of *Ecc* multiplied in the presence of carrot cells. Bacterial populations increased *ca.* 20 fold for both mutant RJP 243 and wild type SCRI 193 in co-culture compared to their controls which increased *ca.* 2 fold. In contrast, mutant RJP 116 increased only 3 fold in co-culture compared to a 1 fold decrease in the control 1/10 th MS medium.

Analysis of variance showed that there were highly significant differences between time, strains, treatments and interactions between time and strains, time and treatment and strains and treatments ($p < 0.01$, Appendix 27). SCRI 193 and RJP 243 multiplied similarly according to Duncan's Multiple Test Range at 5% level. Also it was noticed that in the first 6 hrs, the growth of bacterial strains was the same; only after that time increase in the bacterial number was detected.





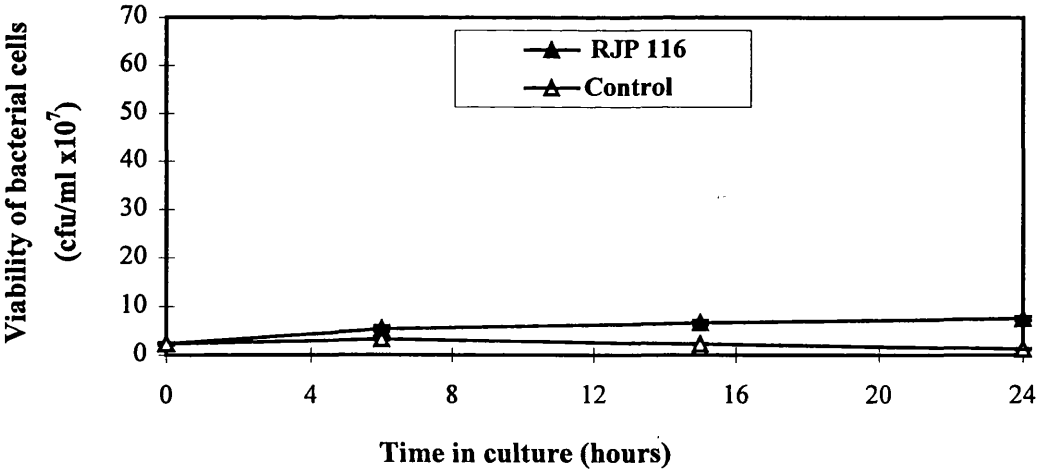


Figure 3.11: Growth of wild type and pectate lyase negative mutants of *Ecc* in co-culture with carrot cv. Morot Duke suspension cells

Each value is the mean and standard deviations of 3 replications.

2.6. THE USE OF MUTANTS TO INVESTIGATE THE MECHANISM OF PATHOGENICITY

2.6.1. Determination of Pectate Lyase Produced by *E. carotovora* and Killing of Carrot Suspension Cells in Co-Culture

Pectic enzymes are released in large quantities by *Erwinia carotovora.* These enzymes are PGL, PG, exo-PGL and oligogalacturonide lyase (OGL) (Collmer et al., 1982). The predominant pectic enzyme secreted by soft rot *Erwinia* spp. is PGL in several isoforms. However, many other components of bacterial pathogenicity, such as cell surface molecules which may be involved in recognition phenomena, are likely to be involved. PGL$^-$ mutants should possess the full complement of these and in the absence of "toxic" PGL, surviving plant cells able to tolerate other components of *Ecc* may be selected.

The use of plant cell suspension culture for the study of plant pathogens facilitates the investigation of individual contributions of the host and pathogen. Defined bacterial mutants offer perhaps the most powerful approach to understanding mechanisms of pathogenicity. The purpose of the study was to determine whether the pathogenic effect (cell killing) of *Erwinia* in carrot suspensions was due to the production of pectic enzymes. It is also possible to use enzyme deficient mutants as an alternative to wild type in screening for disease resistance, since they are unable to secrete extracellular enzymes which are probably the major factors involved in rapid cell killing.

Ten-day-old carrot suspension culture was prepared as described in Materials and Methods, Section 1.4.4 and put in 100 ml conical flasks with 20 ml 1/10 th MS inoculated with 1 ml PCV (after centrifugation at 380×g for 2-3 min).

Ecc mutants supplied from University of Warwick and University of Missouri

(Colombia) were tested separately in two different experiments. *Ecc* strains were prepared as explained in Chapter 2, Section 2.1. The number of bacteria was estimated spectrophotometrically and adjusted to 10^8 cfu/ml. 2.22 ml from 10^8 cfu/ml suspension was inoculated into 20 ml 1/10 th strength MS media to obtain 10^7 cfu/ml at a final concentration. Two replicated flasks were set up for each treatment. The cultures were incubated as explained in Materials and Methods, Section 1.4.4.

Three ml samples were taken from co-cultures. These samples were chilled on ice and the culture supernatants were collected by centrifugation (13000×g for 10 min). The supernatant fluid was saved for assay of PGL and kept on ice. Samples for enzyme assay were taken 2, 5, 10, 20, 24, 48 and 72 hrs after inoculation with bacteria.

2.6.1.1. *Ecc* Mutants (ex. Warwick University)

The reduced virulence mutants of *Ecc* used in the experiment were RJP 116, RJP 243 and PR 54 along with pathogenic wild type SCRI 193. These were tested to find the relationship between their killing ability of carrot cells and PGL production. Pathogenicity tests showed that PR 54, RJP 116 and RJP 243 exhibited reduced virulence while PFP 16 which over-produced pectic enzymes showed increased virulence in carrot taproots (Chapter 3.II, Section 2.2.1). These results support those of Jones et al. (1993) and Reeves et al. (1993).

Figure 3.12(A) demonstrates that inoculation of plant cells with wild type SCRI 193 resulted in statistically significant ($p< 0.01$) cell killing of both single cells and cell units by only 2 hrs (Appendix 28a, 28b). The rate of cell killing then increased up to 15 hrs. No extracellular PGL production was detected until after 10 hrs although cell killing was evident at this time. Over 90% of single cells and cell units were killed by SCRI 193 within 21 hrs.

PR 54 killed the carrot cells slowly compared to wild type SCRI 193. Significant reduction in cell viability occurred 7 hrs after co-culture ($p < 0.01$). The onset of cell killing coincided with PGL production which was evident by 21 hrs. Maximum PGL activity was only 1/10 th that of wild type. It can be seen in Figure 3.12(B) that 15% single cells and cell units were still viable after 24 hrs.

Enzyme deficient mutant RJP 243 was very similar to mutant PR 54 in terms of cell killing. The viability of single cells and cell units was significantly reduced by 5 and 10 hrs respectively. There was no PGL production until 21 hrs, however significant cell killing was evident by 5 hrs. 15% of single cells and 20% of cell units were alive after 24 hrs as shown in Figure 3.12(C).

Low virulence mutant RJP 116 killed the plant cells even more slowly and at lower levels in comparison with the wild type and other mutants and produced no PGL in 24 hrs. As can be seen in Figure 3.12(D), 62% of the cell units and 32% of the single cells were still alive after 24 hrs.

In summary, no mutants produced any PGL until 21 hrs, however significant cell killing was evident by 10 hrs. From these results it appears that a factor(s) other than or in addition to PGL may be involved in pathogenicity.

2.6.1.2. *Ecc* Mutants (ex. Missouri University)

Four mutants of *Ecc* called AC 5010, AC 5013, AC 5017 and AC 5031 and wild type strain *Ecc* 71 were tested.

Assessment of the survival of carrot suspension cultures with all bacterial strains showed that the viability of plant cells was reduced considerably but less killing was caused than by the Warwick mutants. The effect of time and interaction between time and strains on the host cells were also significant ($p < 0.01$, Appendix 29a, 29b).

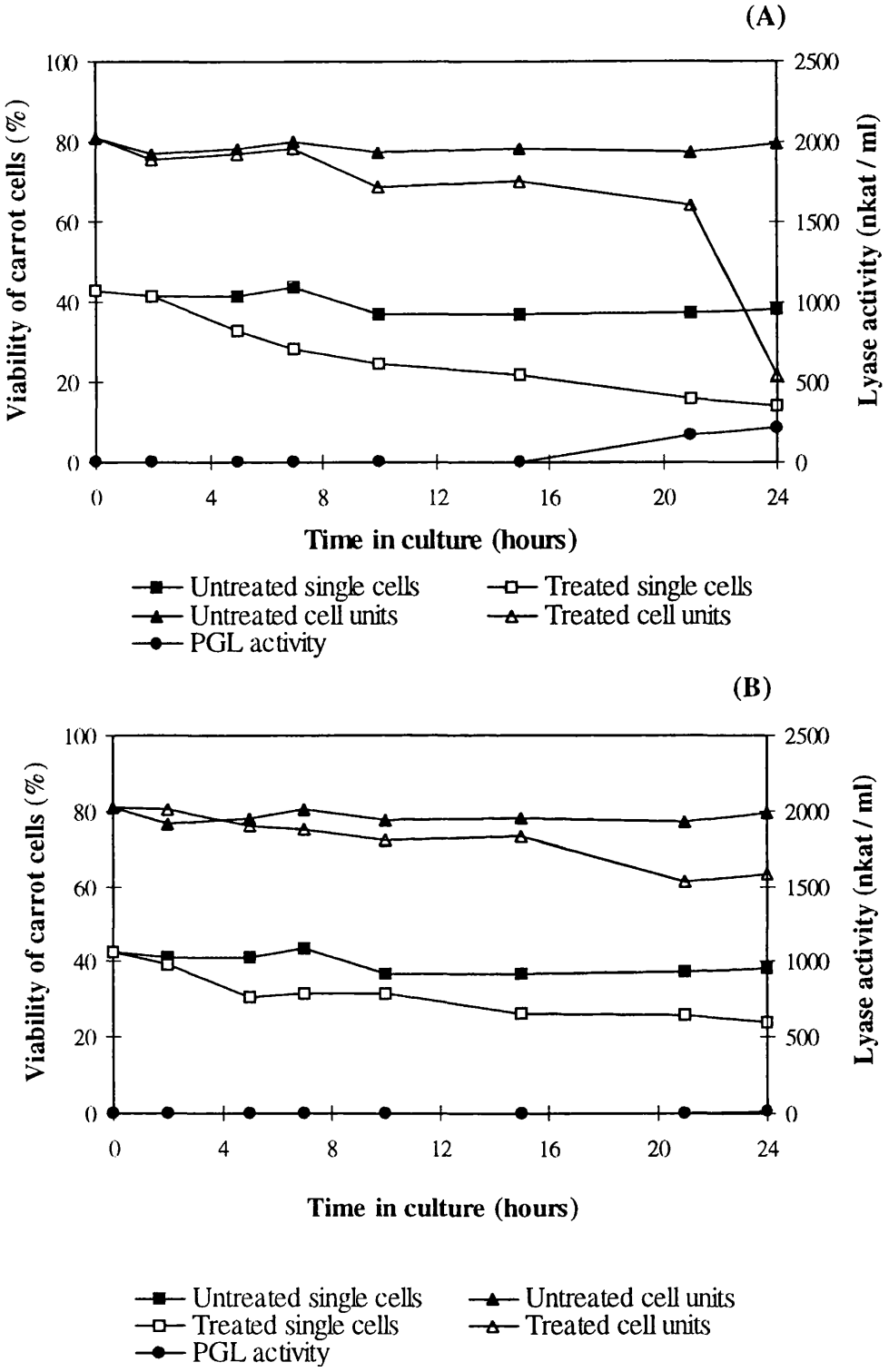
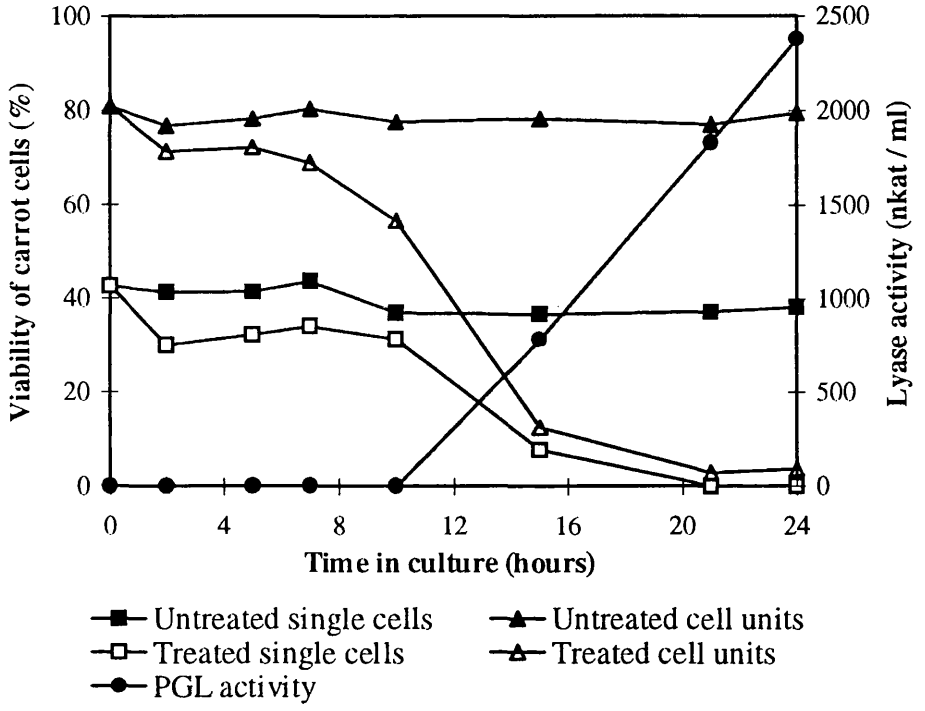


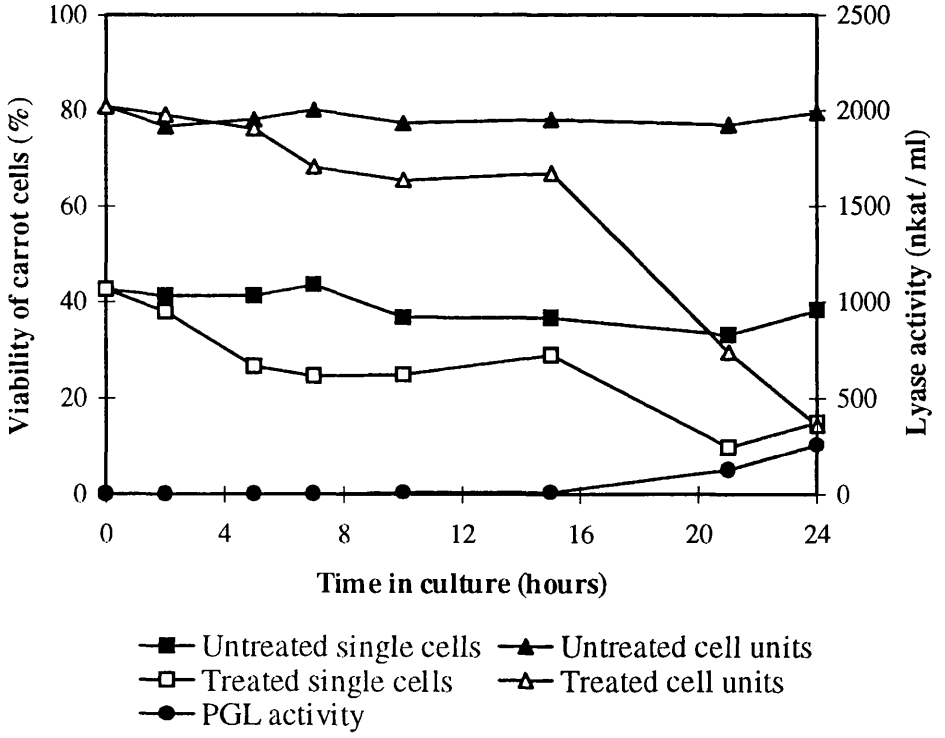
Figure 3.12: The relationship between cell killing and production of pectate lyase by *Ecc* mutants (University of Warwick) (A) RJP 243 (B) RJP 116, (C) Wild type SCRI 193 and (D) PR 54

The analysis of variance results show that the effect of time, strains and interaction between time and strains on the cell killing of carrot suspension cultures were significantly different. Increasing the incubation time of cultures illustrates that pathogenic wild types reduced the viability of the plant cells faster than PGL deficient mutants ($p < 0.01$, Appendix 28a, 28b).

(C)


Viability of carrot cells (%)
Lyase activity (nkat / ml)
Time in culture (hours)
Untreated single cells
Untreated cell units
Treated single cells
Treated cell units
PGL activity

(D)


Viability of carrot cells (%)
Lyase activity (nkat / ml)
Time in culture (hours)
Untreated single cells
Untreated cell units
Treated single cells
Treated cell units
PGL activity

The speed of cell death was very fast with AC 5010 mutant and wild type *Ecc* 71 when compared with other mutants. 6% of cell units and 23% of single cells were found alive within 3 days when plant cells were inoculated with AC 5010 mutant. It was found that there were 30% cells remaining viable in AC 5013, AC 5017 and AC 5031 inoculated suspension cultures even after 4 days. PGL activity was not seen until 12 hrs in both wild type *Ecc* 71 and mutant AC 5010 inoculated suspension cultures. None of these three mutants (AC 5013, AC 5017 and AC 5031) produced PGL at all within 72 hrs and also the reduction of cell viability was slower and remained low (Figure 3.13).

As can be seen in Chapter 3.II, Section 2.2.2, these wild type and enzyme deficient mutants were less pathogenic or non-pathogenic on carrot roots. They were also less aggressive at the cellular level. Therefore, they were not used for future experiments.

2.7. THE EFFECT OF CULTURE FLUIDS FROM CO-CULTURE ON VIABILITY OF SUSPENSION CULTURED CELLS OF CARROT

Previous evidence implicated extracellular PGL as at least one factor responsible for cell killing by *Ecc*. In order to test this hypothesis, toxicity of sterile fluids from co-cultures were determined. Toxicity of fluids could be related to PGL by assaying for enzyme activity during co-culture. Wild type SCRI 193 and enzyme deficient mutant RJP 116 were used for these experiments.

Ten-day-old carrot cells and bacterial suspensions were prepared as described in Materials and Methods, Section 1.4.4 and 2.1. Five ml, 10^8 cfu/ml bacterial suspension was inoculated into 250 ml conical flasks containing 45 ml 1/10 th MS with 2.5 ml PCV

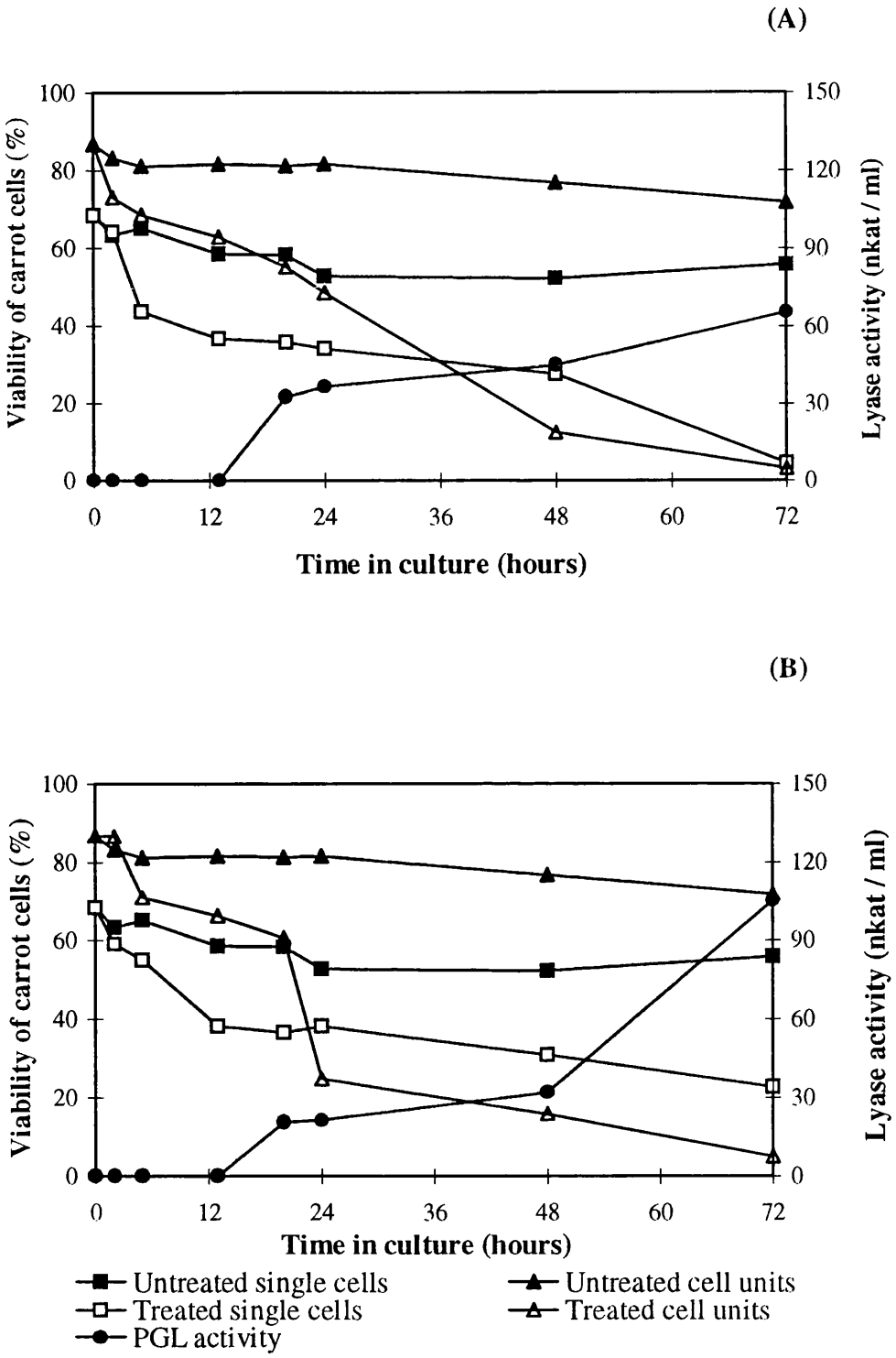
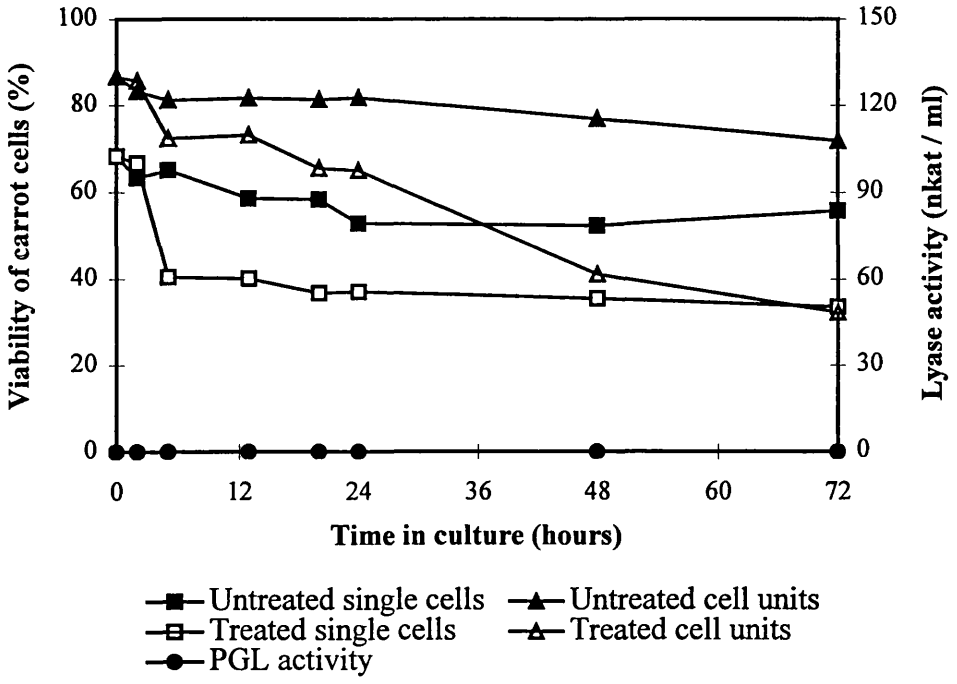
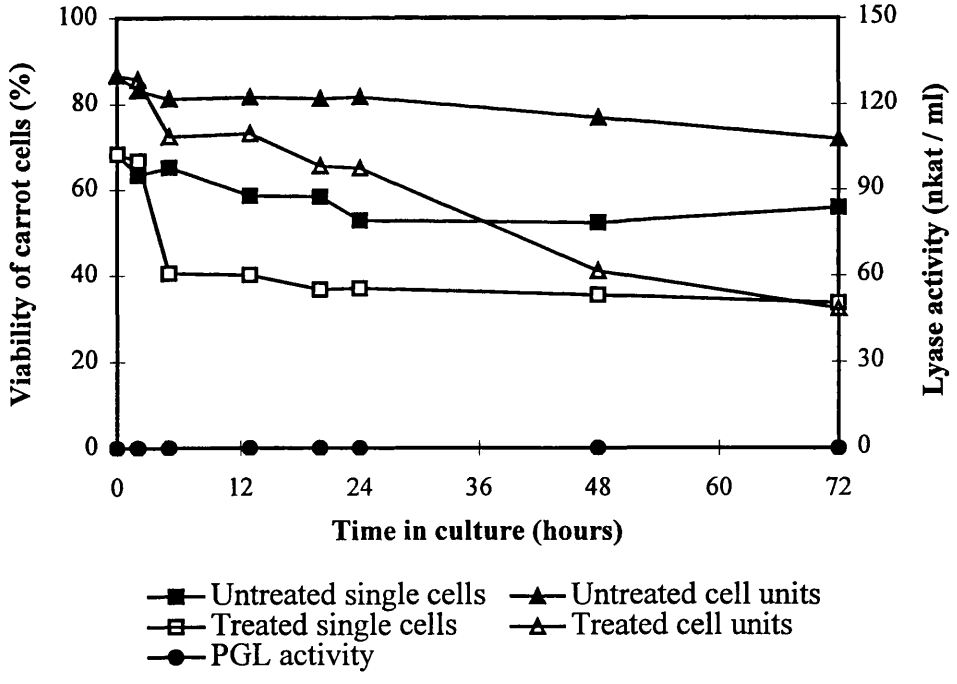


Figure 3.13: The relationship between cell killing and production of pectate lyase by *Ecc* mutants obtained from University of Missouri (A) Wild type *Ecc* 71, (B) AC 5010, (C) AC 5013, (D) AC 5017 and (E) AC 5031

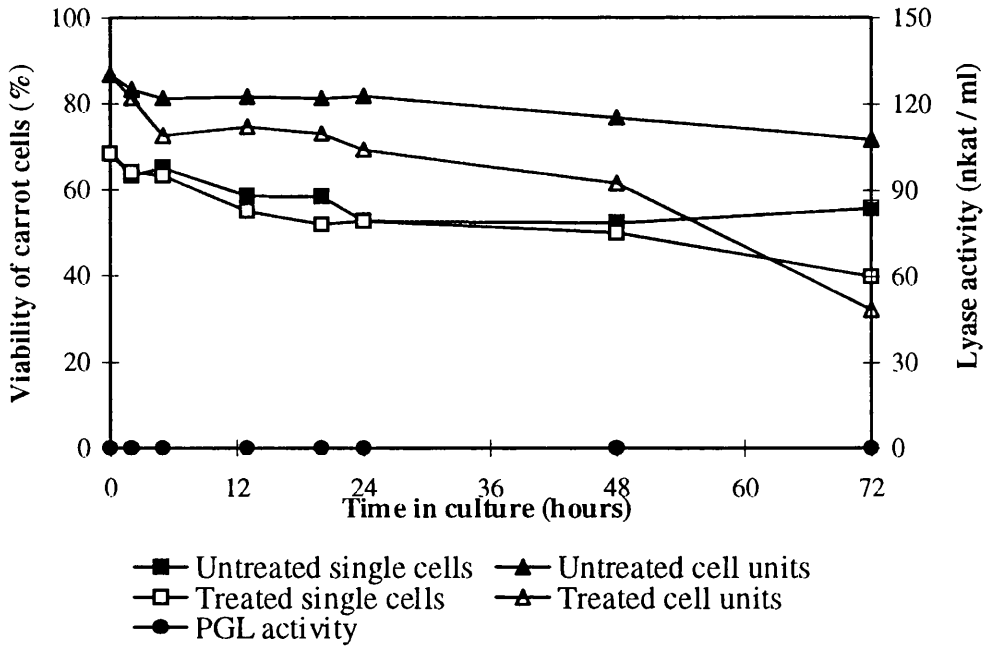
(C)


Viability of carrot cells (%)
Lyase activity (nkat / ml)
Time in culture (hours)
Untreated single cells
Untreated cell units
Treated single cells
Treated cell units
PGL activity

(D)


Viability of carrot cells (%)
Lyase activity (nkat / ml)
Time in culture (hours)
Untreated single cells
Untreated cell units
Treated single cells
Treated cell units
PGL activity

(E)



(after centrifugation at 380×g for 2-3 min) plant cells to obtain a final concentration of 10^7 cfu/ml and these were cultured as described in Materials and Methods, Section 1.4.4. Culture fluids were collected at time intervals and kept at 4 °C. Culture supernatants were then centrifuged at 4000×g for 30 min and filter-sterilised through a membrane filter of pore size 0.2 μm (Sartorius Ltd.). After sterilisation, culture fluids were checked whether there were any bacteria in the fluids. 22.2 ml of this medium was placed into 100 ml conical flasks containing 1 ml PCV carrot cells. Two replicated flasks were set up for each treatment. PGL activity of the culture fluids was assayed as described in Materials and Methods, Section 6.1.

2.7.1. Culture Fluids from Wild Type

Culture fluids containing PGL were found to correlate with toxicity towards carrot cells whereas culture fluids containing barely detectable PGL were non-toxic. Thus, culture fluids from the host-pathogen interaction after either 27 or 36 hrs, contained high PGL and displayed significant cell killing (85%) on carrot cell units within 2 hrs of application. However, culture fluids from 6 hrs contained very low amount of PGL and did not reduce the viability of carrot cells. No significant differences were found between control and 6-hour-culture fluids on cell killing of cell units until 20 hrs when there was a significant difference between control and single cells (Appendix 30a, 30b). As can be seen in Figure 3.14, cell units were far more sensitive to extracellular fluids than single cells.

2.7.2. Culture Fluids from Enzyme Deficient Mutant

PGL was obtained from enzyme deficient mutant RJP 243. Extracellular PGL of RJP 243 was absent at 7 hrs but was detectable at 15 hrs and about 25% of wild type SCRI 193 by 24 hrs. No significant reduction was observed in the viability of cell units in 2 days while single cells displayed a significant reduction by 4 hrs (Duncan's Multiple Range Test at 5%). The mortality of plant cell units was reduced by 1-4% compared to 22% of single cells in 24 hrs (Figure 3.15).

The effect of time was highly significant on the killing of both single cells and cell units ($p < 0.01$, Appendix 31a, 31b). Different culture fluids also had very different effects ($p < 0.01$) and interaction between time and culture fluids was significant ($p < 0.01$, Appendix 31a, 31b) on single cells while no significant difference was found between culture fluids and time on the viability of plant cell units.

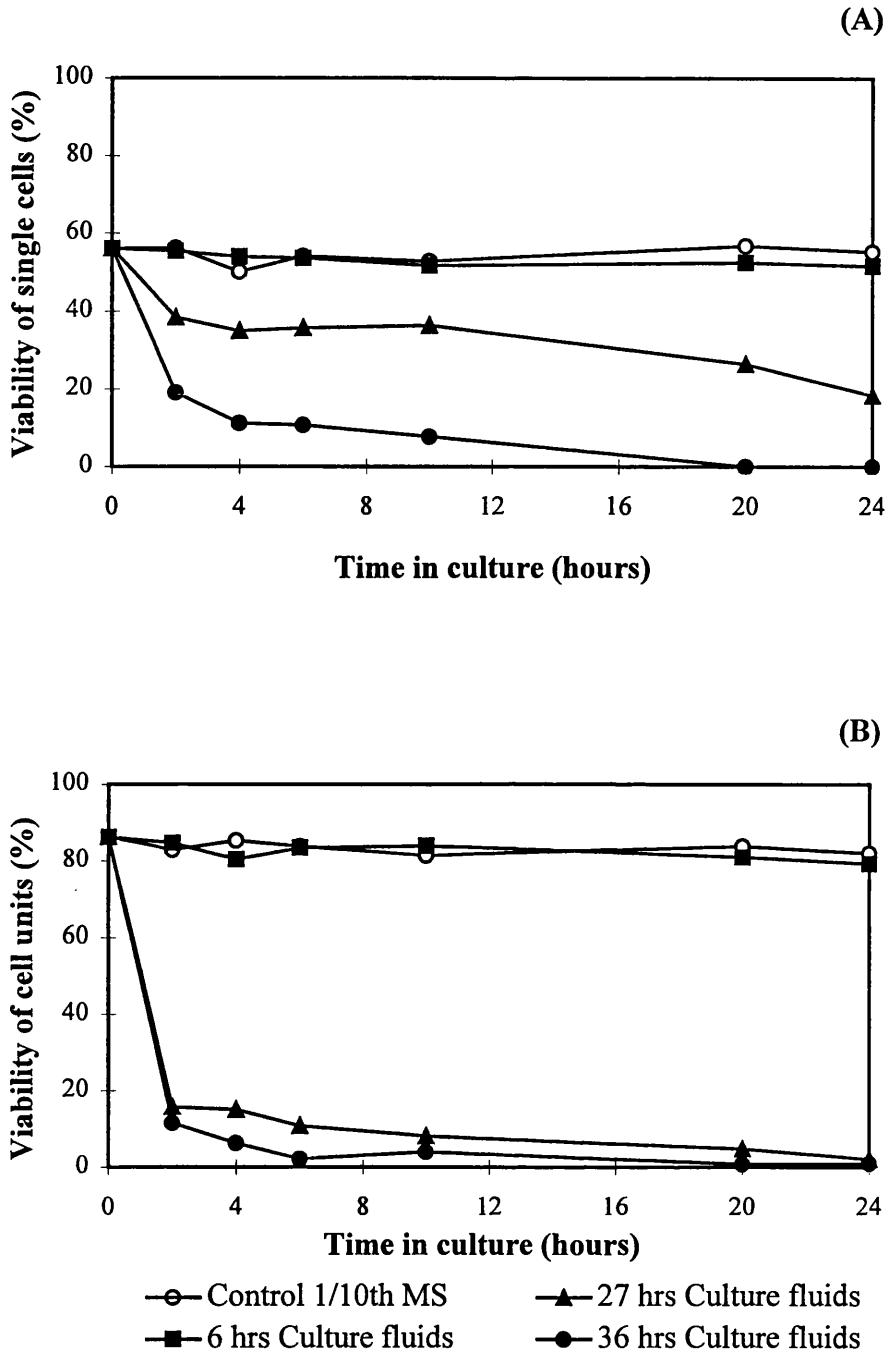


Figure 3.14: The effect of culture supernatants obtained from wild type SCRI 193 on suspension cultured single cells (A) and cell units (B) of carrot cv. Morot Duke

PGL activity of culture fluids was calculated from the mean of 2 replicated enzyme assays and can be given in μkat as; 6 hrs: 0.00275, 27 hrs: 0.471, 36 hrs: 1.74. Analysis of variance results presented that time, culture fluids and interaction between time and culture fluids were significantly different on the killing of carrot cells ($p < 0.01$, Appendix 30a, 30b).

It may be concluded that extracellular fluids of enzyme deficient mutant RJP 243 were non-toxic to cell units and should only have a minimal effect on single cells. However, single cells were more sensitive to low amount of PGL than cell units. Thus no clear evidence for production of an extracellular toxic factor other than PGL was obtained; it is feasible that any such bacterial "toxin" could be cell associated.

2.8. GROWTH AND PRODUCTION OF PGL BY *Ecc* WILD TYPE IN AXENIC CULTURE

The object of this experiment was to determine whether PGL of *Ecc* induced differently in axenic culture was toxic to carrot embryogenic suspension cultures.

Five ml of the wild type *Ecc* strain SCRI 193 (10^8 cfu/ml) was inoculated into 45 ml of the Minimal Salt Medium containing either glucose (1%) or pectate (1%) as carbon source (Stack et al., 1980) to give a final concentration of 10^7 cfu/ml. The media were adjusted to pH 7.0 and bacterial cultures were placed in a shakers at 30 °C for 48 hrs in dark. Culture fluids were then collected, centrifuged at 13000×g, 0 °C for 30 minutes and were dialysed overnight in distilled water (pH 7.0).

Carrot cv. Morot Duke cells from 10-day-old cultures were prepared as described in Materials and Methods, Section 1.4.4. Five ml samples containing 2.5 ml PCV plant cells were placed in 250 ml conical flasks with 45 ml 1/10 th strength MS.

The treatments used in this experiment were fluids from: i) bacteria grown in medium with glucose as carbon source; ii) bacteria grown in medium with pectate as carbon source to induce PGL synthesis (a dilution series of 1/2, 1/10, 1/100 and 1/1000) was used; iii) heat inactivated fluids from bacteria grown in medium with pectate as carbon source. Cultures were placed in a 100 rpm shaker at 25 ± 1 °C.

The pectate cultures contained PGL at 2.94 μkat which is about 1.5 fold that obtained in co-culture; glucose cultures contained no detectable PGL.

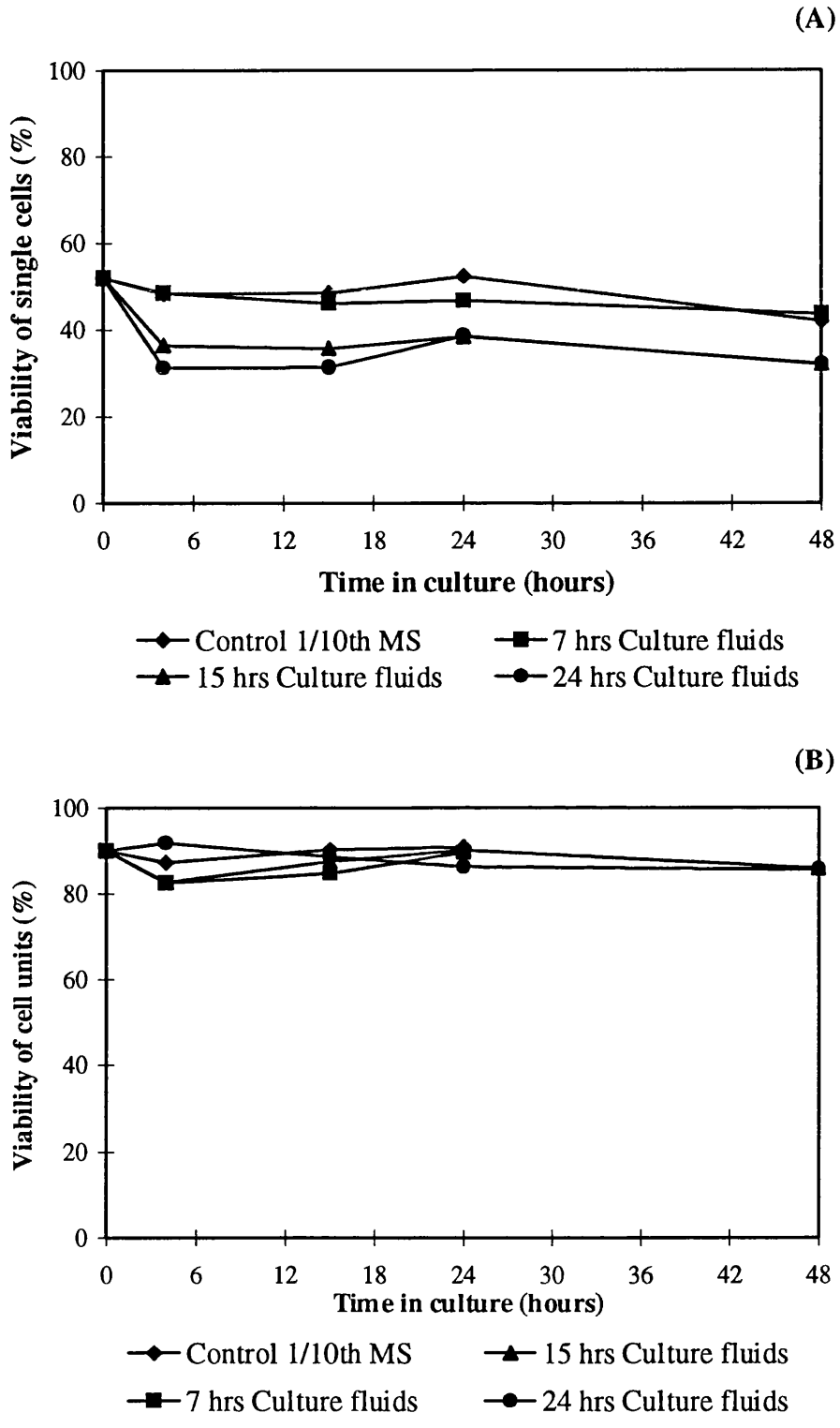


Figure 3.15: The effect of culture supernatants obtained from enzyme deficient mutant RJP 243 on suspension cultured single cells (A) and cell units (B) of carrot cv. Morot Duke

PGL activity of culture fluids was calculated from the mean of 2 replicated enzyme assays and can be given in µkat as, 7 hrs: 0, 15 hrs: 0.0175, 24 hrs: 0.119.

Figure 3.16 illustrates that neither the heat inactivated pectate fluids, glucose culture supernatants nor 1/1000 pectate fluids affected cell viability of carrot single cells. Only 1/2 strength pectate fluids were effective on single cells but there were still *ca.* 10% viable cells by 2 days. Carrot cell units were more sensitive to PGL than single cells. 1/2, 1/10 and 1/100 strength pectate containing medium caused *ca.* 50%, 30% and 20% cell-unit killing in 15 minutes which increased to *ca.* 93%, 65% and 55% respectively by 8 hrs. Plant cells were killed only slowly and at low levels by 1/100 strength pectate fluids. PGL activity at 1/10 pectate dilution was approximately equivalent to that of co-culture fluids which were obtained from wild type SCRI 193 after 27 hrs (see Section 2.7.1). 0.3-0.4 μkat PGL was sufficient to reduce the viability of carrot single cells and cell units down to 15-25% in 2 hrs. As can be seen in Figure 3.16, not all carrot cells were killed even at highest activity. It can be concluded that PGL is the likely major component responsible for cell killing.

2.9. THE USE OF ANTIBIOTICS FOR KILLING BACTERIA FROM CO-CULTURED PLANT CELLS TO REGENERATE PLANTS

The purpose of the experiments was to examine the sensitivity of *Ecc* wild type and PGL$^-$ mutants and plant cells to various antibiotics to be used for regeneration from embryogenic units challenged in co-culture.

2.9.1. The Effect of Antibiotics on Growth of *Ecc* Wild Type and Pel$^-$

Antibiotics used in this experiment were ampicillin (5, 25 and 50 μg/ml), chloramphenicol (2.5, 10 and 20 μg/ml), cycloheximide (5, 25 and 100 μg/ml), gentamicin (10, 25 and 50 μg/ml), kanamycin (5, 10 and 25 μg/ml), rifampicin (15, 25

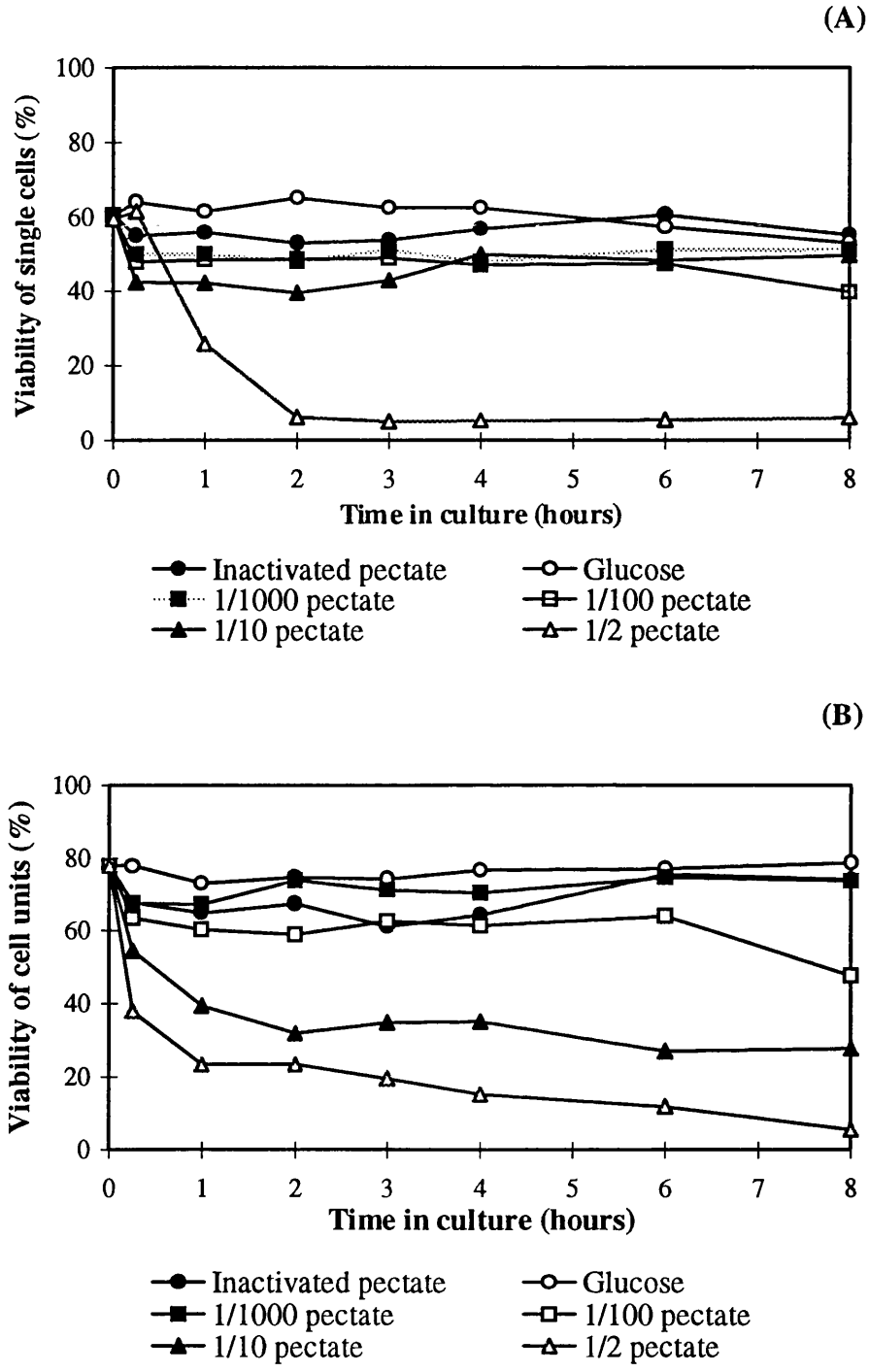


Figure 3.16: Effect of culture supernatant on suspension cultured single cells (A) and cell units (B) of carrot cv. Morot Duke

PGL activity of undiluted culture fluids was 2.94 µkat and that of glucose culture fluids was zero. There were significant differences between time, treatment and interaction between time and treatment on carrot cell units ($p < 0.01$, Appendix 32). Mean separation among all values were done by Duncan's Multiple Range Test (5%). The results show that 1/1000 pectate, heat inactivated pectate and glucose fluids used as control, had a similar effect on the killing of carrot cell units.

and 50 µg/ml) and streptomycin (25, 50 and 100 µg/ml). Stock antibiotics at 25 mg/ml were prepared freshly whenever they were needed.

The antibiotic sensitivity test was carried out on semi-solid LB agar (12 g/l) containing different concentrations of antibiotics. LB and NA semi-solid media without antibiotics were included as controls. One wild type (SCRI 193) and two enzyme deficient mutants (RJP 116, RJP 243) were tested. 100 µl of 10^4 cfu/ml was spread onto the 5 petri dishes for each treatment. Petri dishes were placed at 28 ± 2 °C for 2 days.

It can be seen from Table 3.34 that chloramphenicol, kanamycin and gentamicin inhibited bacterial growth when at ≤ 10 µg/ml. Ampicillin inhibited growth at 5 µg/ml. Cycloheximide and rifampicin were ineffective. Streptomycin mainly controlled growth at ≥ 25 µg/ml.

2.9.2. The Effect of the Antibiotics on the Viability of Carrot Cells

Based on their efficacy vs. *Ecc* the antibiotics tested were kanamycin, streptomycin, gentamicin, chloramphenicol and rifampicin at 25 µg/ml and 50 µg/ml concentrations.

Carrot cv. Morot Duke cells were prepared as described in Materials and Methods, Section 1.4.4. Ten ml of 1/10 th MS medium with above concentrations of antibiotics were placed into 50 ml conical flasks containing 10-day-old (0.5 ml PCV) carrot cells. 1/10 th MS medium without antibiotics was used as control. Two replicated conical flasks for each treatment were set up and incubated as described in Materials and Methods, Section 1.4.4.

Figure 3.17 reveals that rifampicin killed the majority of single cells and cell units within 24 hrs while streptomycin, gentamicin, kanamycin and chloramphenicol

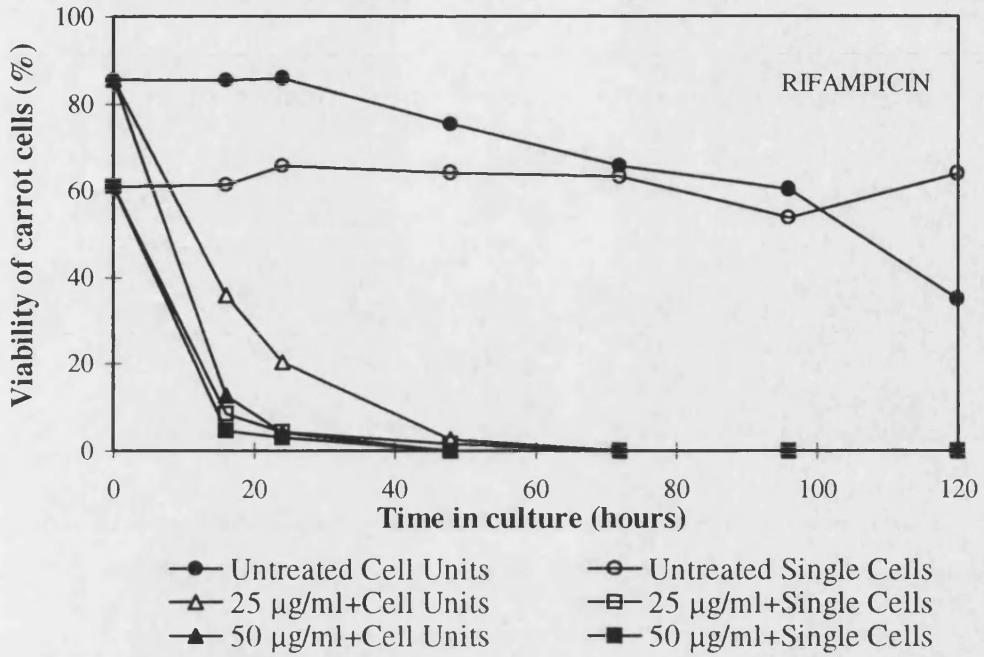
Table 3.34: Effects of antibiotics on growth of *Ecc* wild type and Pel⁻ mutants

Antibiotics	Concentration (μg/ml)	Bacterial strains		
		SCRI 193 Wild Type	RJP 116 Pel⁻	RJP 243 Pel⁻
chloramphenicol	2.5	+	+	+
	10	-	-	-
	20	-	-	-
ampicillin	5	-	-	-
	25	-	-	-
	50	-	-	-
kanamycin	5	*	+	*
	10	-	-	-
	25	-	-	-
gentamicin	10	-	-	-
	25	-	-	-
	50	-	-	-
streptomycin	25	-	-	*
	50	-	-	-
	100	-	-	-
rifampicin	15	+	+	+
	25	+	+	+
	50	*	+	+
cycloheximide	5	+	+	+
	25	+	+	+
	100	+	+	+
Control (NA)		+	+	+
Control (LB)		+	+	+

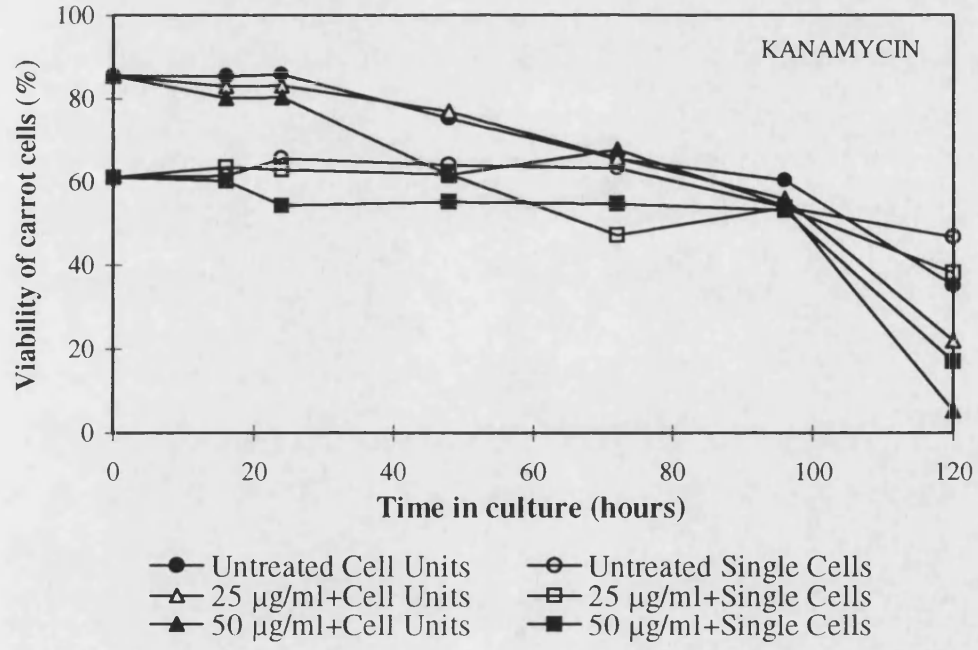
Growth index :
- : no growth
* : weak growth
+ : good growth

did not affect viability very much at tested concentrations up to 2 days. It might be possible to remove *Ecc* from co-culture with less than 2 days exposure to antibiotic. Therefore, it may not be necessary to determine such long time courses for viability measurements.

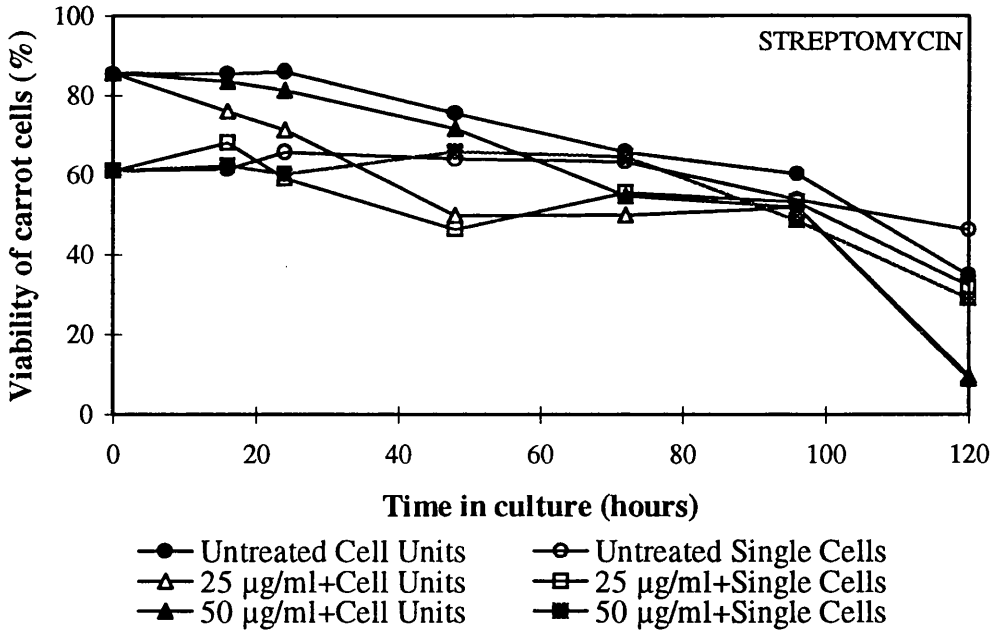
(A)


RIFAMPICIN
Viability of carrot cells (%)
100
80
60
40
20
0
0
20
40
60
80
100
120
Time in culture (hours)
Untreated Cell Units
Untreated Single Cells
25 µg/ml+Cell Units
25 µg/ml+Single Cells
50 µg/ml+Cell Units
50 µg/ml+Single Cells

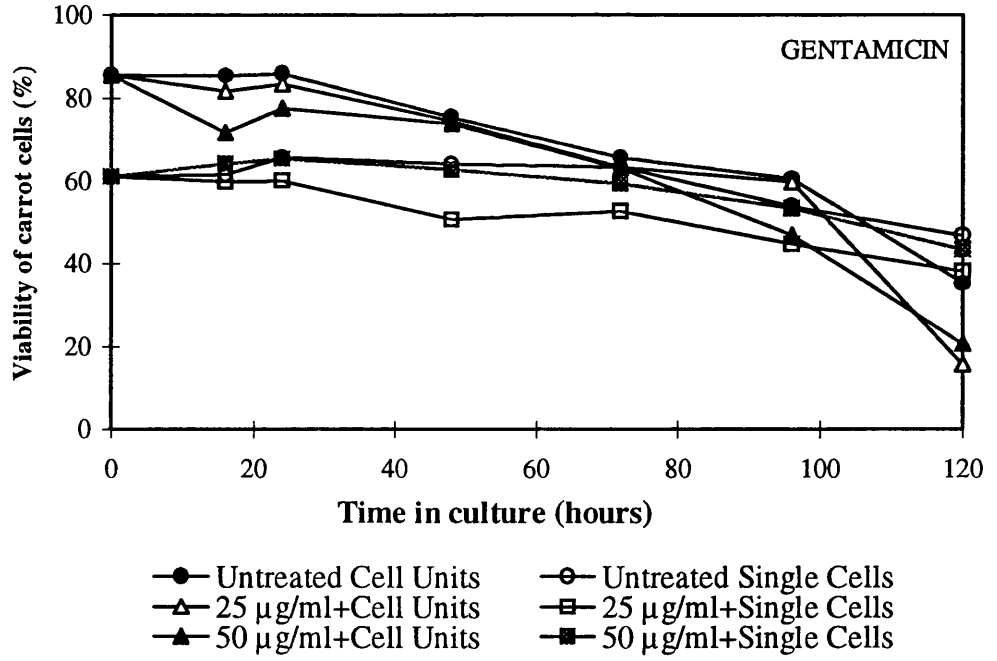
(B)


KANAMYCIN
Viability of carrot cells (%)
100
80
60
40
20
0
0
20
40
60
80
100
120
Time in culture (hours)
Untreated Cell Units
Untreated Single Cells
25 µg/ml+Cell Units
25 µg/ml+Single Cells
50 µg/ml+Cell Units
50 µg/ml+Single Cells

(C)


STREPTOMYCIN
Viability of carrot cells (%)
100
80
60
40
20
0
0
20
40
60
80
100
120
Time in culture (hours)
Untreated Cell Units
Untreated Single Cells
25 µg/ml+Cell Units
25 µg/ml+Single Cells
50 µg/ml+Cell Units
50 µg/ml+Single Cells

(D)


GENTAMICIN
Viability of carrot cells (%)
100
80
60
40
20
0
0
20
40
60
80
100
120
Time in culture (hours)
Untreated Cell Units
Untreated Single Cells
25 µg/ml+Cell Units
25 µg/ml+Single Cells
50 µg/ml+Cell Units
50 µg/ml+Single Cells

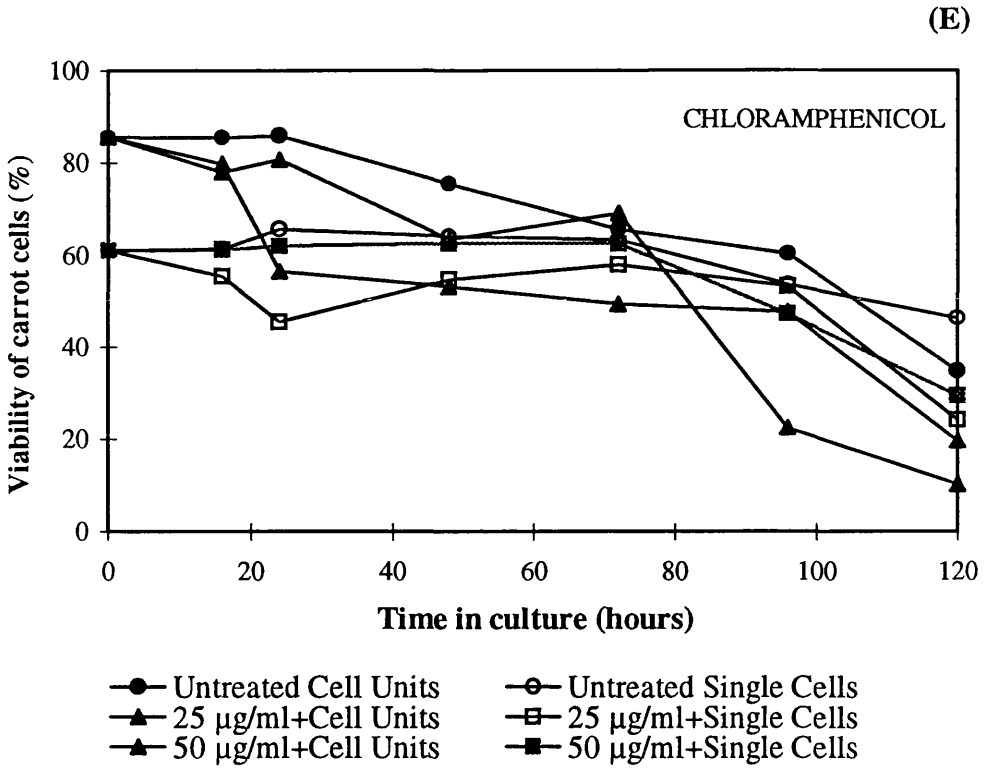


Figure 3.17: The effect of antibiotics (A) rifampicin, (B) kanamycin, (C) streptomycin, (D) gentamicin and (E) chloramphenicol on the embryogenic carrot suspension cultures

There was positive correlation between carrot cell killing and incubation time. Antibiotic treated single cells and cell units reduced the viability significantly when the time in culture increased ($p < 0.01$, Appendix 33a, 33b).

2.10. DETERMINATION OF ANTIBIOTICS FOR KILLING OF BACTERIA AND REGENERATION OF PLANTLETS FROM CO-CULTURED PLANT CELLS WITH WILD TYPE SCRI 193

The aim was to examine the possible elimination of *Ecc* cells by antibiotics from co-culture after challenging plant cells with bacteria. The tolerance of plant cells to antibiotics after co-culture treatment may not be the same as that found in Section 2.9.2 since the plant cells will have been stressed by exposure to bacteria.

Ten-day-old carrot cv. Morot Duke cells maintained for two months in MS2 supplemented with 0.5 µM 2,4-D liquid media and bacterial cells were prepared as explained in Materials and Methods, Section 1.4.4 and in Section 2.1 respectively. Carrot cells at 1 ml PCV per 100 ml conical flasks containing 20 ml 1/10 th MS medium and

Ecc wild type SCRI 193 at 10^7 cfu/ml were set up for co-culture treatment. Two conical flasks were established.

Carrot cells were challenged with bacteria for 15 hrs until the viability of cell units was reduced to 25-30%. After the co-culture period, the majority of bacteria were removed from culture fluids by washing the plant cells with 75 ml 1/10 th MS medium. This was achieved by sieving them through a 22.4 μm pore nylon sieve and then washing again with 25 ml 1/10 th MS medium by centrifugation at 200 rpm for 2-3 min. The washed cells were placed into 50 ml conical flasks containing 10 ml, 1/10 th MS medium with various antibiotics. The antibiotics tested were kanamycin, gentamicin, streptomycin and chloramphenicol at 25 μg/ml.

It can be seen from Table 3.35 that the bacterial population proliferated 10 times during the 15 hrs co-culture and that bacterial number was then reduced almost 30 times by washing of plant cells after co-culture. Kanamycin and streptomycin were extremely effective in controlling bacterial growth. Both antibiotics killed bacteria within 15 hrs treatment. Gentamicin and chloramphenicol were ineffective at this time. In previous experiment (Figure 3.17), streptomycin (25 μg/ml) reduced viability of cell units by 10% after 15 hrs. It appears that kanamycin could be used for regeneration experiments.

2.11. REGENERATION OF MATURE SOMATIC EMBRYOS AFTER CO-CULTURE TREATMENT

Carrot cells were challenged with bacteria and then treated with antibiotics to select possible resistant cells from co-culture as explained in Section 2.10. Kanamycin was chosen to remove bacteria from co-cultures as it did not reduce the viability of the plant cells and killed the bacterial cells in 15 hrs.

Table 3.35: Determination of number of bacteria from challenged plant cells and effects of antibiotics

Time in culture (hrs)	Applied treatments	The number of bacteria 10^6 cfu/ml
0	**-—**	21.9 ± 0.91
15	**Co-culture**	248 ± 37.7
15.5	**Washing**	8.19 ± 1.5
2	**kanamycin**	1.6 ± 0.17
	streptomycin	6.93 ± 0.34
	gentamicin	9.68 ± 1.03
	chloramphenicol	1.50 ± 0.27
5	**kanamycin**	0.000044 ± 0.000003
	streptomycin	2.43 ± 0.17
	gentamicin	12.5 ± 1.9
	chloramphenicol	3.25 ± 0.2
10	**kanamycin**	0.000003 ± 0
	streptomycin	0.000030 ± 0.0000015
	gentamicin	34.67 ± 2.06
	chloramphenicol	1.44 ± 0.076
15	**kanamycin**	0
	streptomycin	0
	gentamicin	43.1 ± 4.38
	chloramphenicol	2.34 ± 0.1
24	**kanamycin**	0
	streptomycin	0
	gentamicin	0.000224 ± 0.000026
	chloramphenicol	1.07 ± 0.04

Data are the mean and standard deviations of 3 replicated NA plates. Ten-day-old carrot cells were challenged with *Ecc* for 15 hrs. Various antibiotics were used to remove bacteria after co-culture treatment. Antibiotics were added at 25 µg/ml.

1/10 th MS and 1/10 th MS supplemented with 25 µg/ml kanamycin media were used as controls to determine the effect of kanamycin on the regeneration capacity of carrot cells. The same procedure except bacteria was followed for control treatments.

After co-culture (15 hrs) and kanamycin treatments (24 hrs), challenged cells were returned to carrot suspension culture medium (MS2 + 5 µm 2,4-D) and were subcultured every week into the fresh medium for a total of 28 days. Half ml aliquots of 28-day-old suspension culture (*ca.* 850-1000 cells/0.5 ml) were then placed into 9 cm petri dishes with MS2 media in 10 drops per plate. Three replicated plates were incubated in the culture room at 25 ± 1 °C. One month after incubation time, green cotyledonary stage embryos were counted.

Table 3.36: Regeneration of mature embryos from embryogenic units after co-culture treatment with wild type SCRI 193

Treatments	Number of regenerants (Somaclone/ml)
Control (1/10 MS)	244.3 ± 74.44
kanamycin treated control (1/10 MS + kanamycin)	11.2 ± 6.8
Co-cultured plant cells	0

Data are the mean and standard deviation of 3 replicated plates. Carrot cells were challenged with bacteria for 15 hrs. Kanamycin at 25 μg/ml was applied for 24 hrs to remove the bacteria.

Kanamycin seriously reduced the regeneration capacity of the plant cells 20 times at the concentration used (Table 3.36). No regenerant from co-culture was obtained (Plate 29). Co-culture apparently further reduced the regeneration capacity to a value of zero.

3. CO-CULTURES OF PLANT AND FUNGAL CELLS

3.1. CO-CULTURE OF CARROT CELLS AND *P. violae*

Co-culture of carrot cells and *P. violae* was attempted with the aim of regenerating plants with increased disease resistance or tolerance from surviving cells. It was considered necessary to obtain *P. violae* as small, washed, mycelial fragments in order to facilitate good contact with carrot suspension cells in shake culture. The "pathogenicity" of *Pythium* spp. *in vitro* has not previously been investigated.

P. violae was grown on DowElanco medium containing glucose as carbon source. Five 250 ml conical flasks with 100 ml of above medium were inoculated with 10, 0.5 cm^2 plugs taken from the edge of V8 plates using a cork borer. Flasks were shaken (160 rpm) at 20 ± 1 °C in the dark for 14 days. The mycelia were then collected and growth was estimated by the measurement of fresh weight. Contents of two conical

Plate 29: Regeneration of mature somatic embryos from embryogenic units challenged in co-culture. Note: kanamycin at 25 μg/ml strongly inhibited proliferation and regeneration of carrot cells. Control (1/10 MS) on the right, kanamycin treated control (1/10 MS + kanamaycin) in the middle and co-cultured plant cells on the left

Plate 30: Regeneration of green cotyledonary stage somatic embryos from 7-month-old carrot cv. Morot Duke suspension cultures on solid MS2 and MS2 supplemented with 1% activated charcoal media. Note: activated charcoal did not affect embryo formation

Plate 29

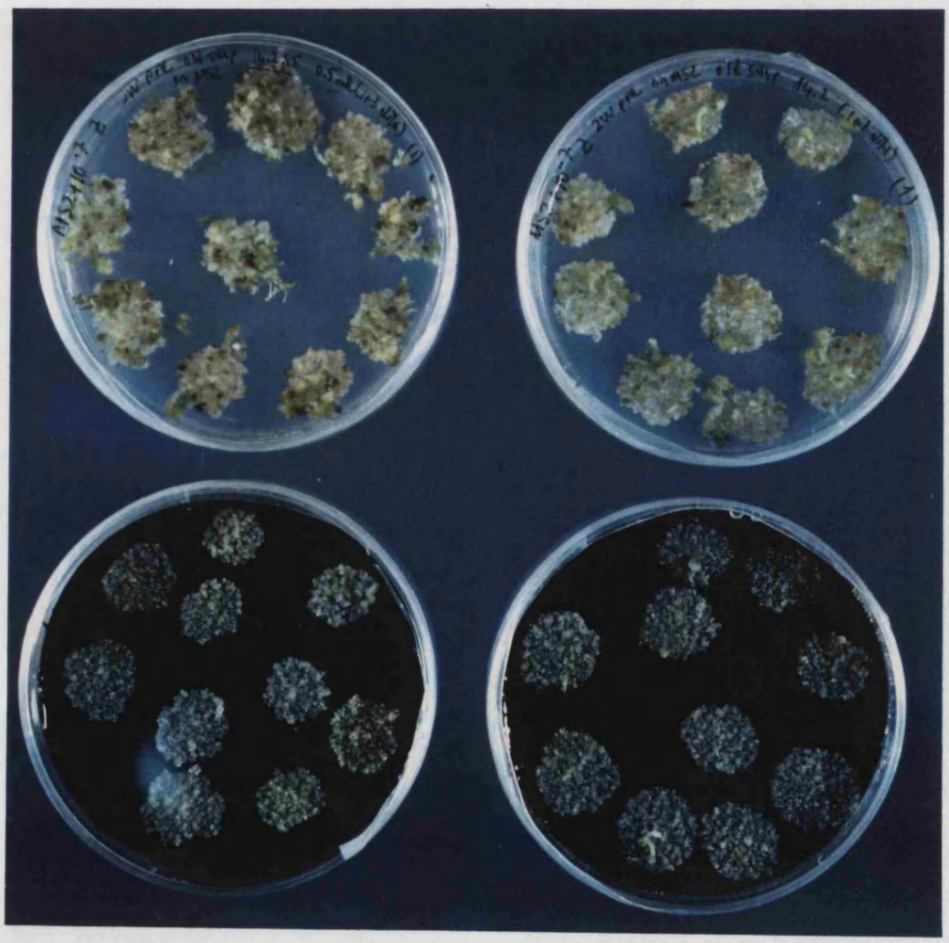


Plate 30

flasks were sieved through 1000 µm pore sieve. The mycelia trapped on the 1000 µm sieve were taken aseptically and comminuted with a hand homogeniser for 10-15 min and then sieved through 500 µm pore sieve. Finally, mycelial fragments which passed through 500 µm pore sieve were collected by centrifugation (4000×g, 15 min). DowElanco medium was replaced with 1/10 th MS medium. Carrot cells were prepared as explained in Chapter 2, Section 1.4.4 and inoculated at a final density of 1 ml PCV per 100 ml conical flasks with 20 ml of the 1/10 th MS test medium. The amounts of mycelium treated in the co-culture were 1 ml, 2 ml and 4 ml PCV (670×g for 10 min). There were two replicate flasks for each treatment. Co-cultured carrot cells with *P. violae* were incubated in a shaker at a speed of 100 rpm at 20 ± 1 °C in light (30 µmol m^{-2} s^{-1}). Co-culture of carrot cells and small mycelium fragments of *P. violae* are shown in Plate 31 and Plate 32.

Co-culture with either 1 ml, 2 ml or 4 ml PCV of *P. violae* resulted in complete death of cells of cultivar Morot Duke within 48 hrs. All treatments with *P. violae* caused almost 60% of carrot cell death by 24 hrs (relative to controls). At this time the viability of the cell unit controls was 90% and single cell controls was 50% and differed significantly ($p < 0.01$, Appendix 34a, 34b) from cultures with *P. violae* (Figure 3.18).

Viability of single carrot cells did not change within 3 hrs when 1 and 2 ml PCV were used but, there was significant differences when 4 ml PCV was used at this time (Duncan's Multiple Range Test at 5% level). However, after this time the speed of killing of single cells increased rapidly. All treatments (1, 2 and 4 ml PCV) tested significantly killed cell units of carrot suspension cultures by 3 hrs. However, the effect of 1 ml and 2 ml PCV of *P. violae* on the killing of cell units was similar to that of 4 ml PCV.

Plate 31: Co-cultures of carrot cells and *P. violae*. The white arrows show mycelia of *P. violae* and black arrows show embryogenic cells (×175)

Plate 32: Detail of co-cultures of carrot cells and *P. violae*. The white arrows show mycelia of *P. violae* and black arrows show embryogenic cells (×350)

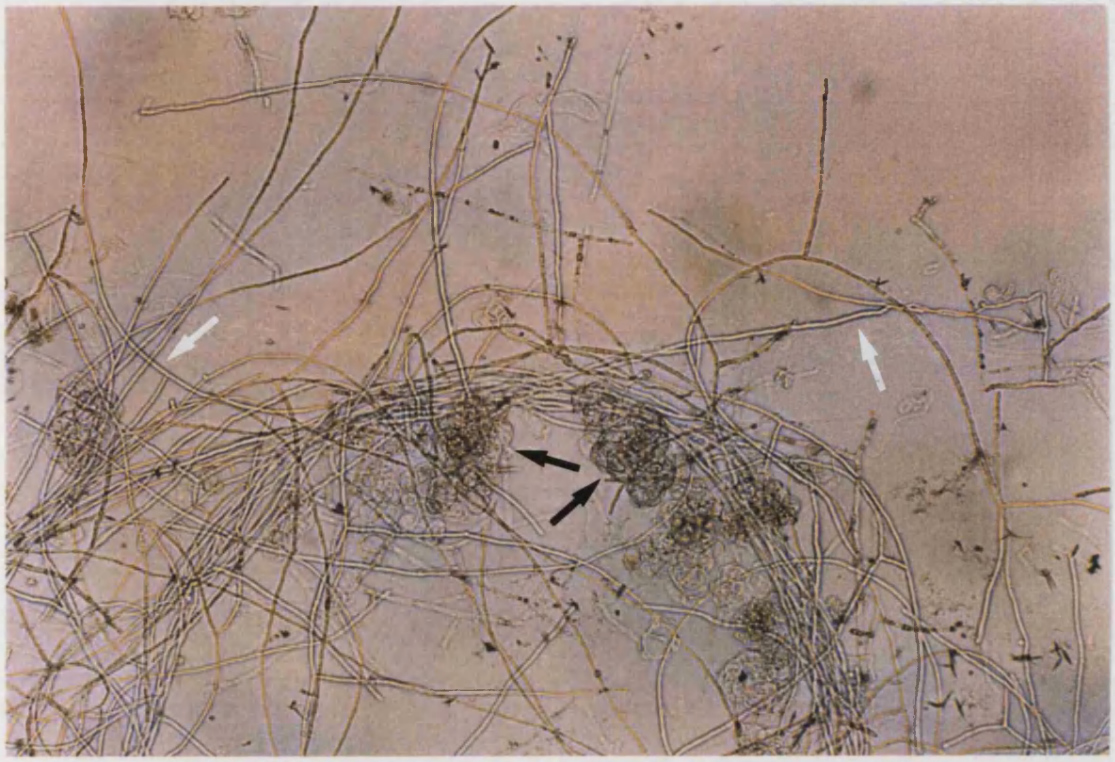


Plate 31

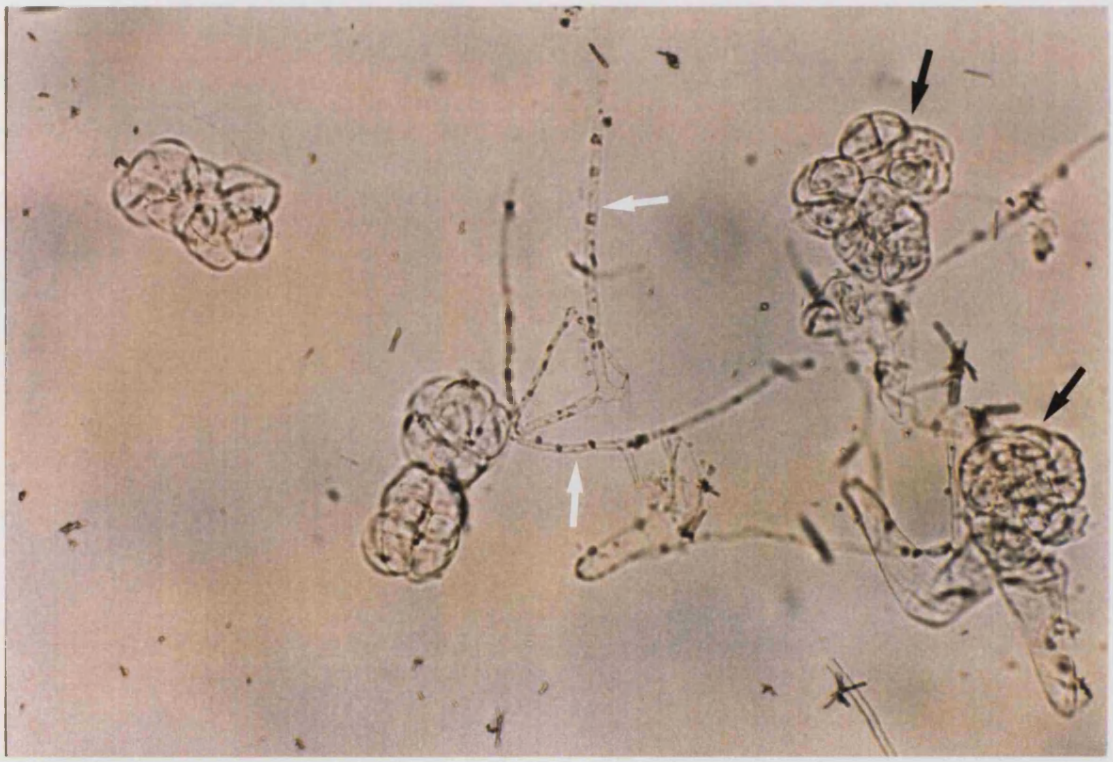
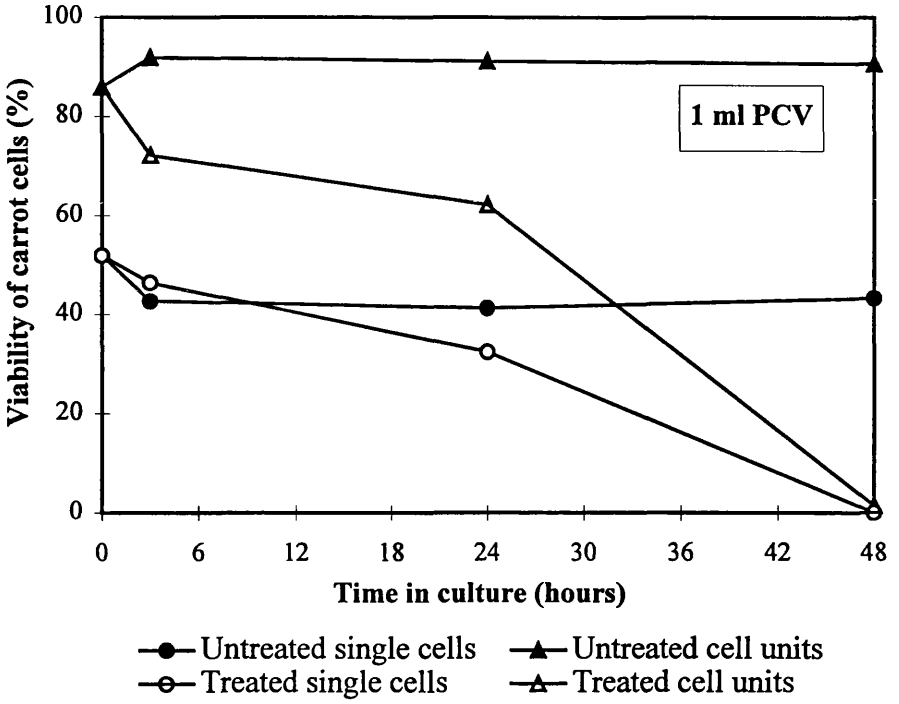
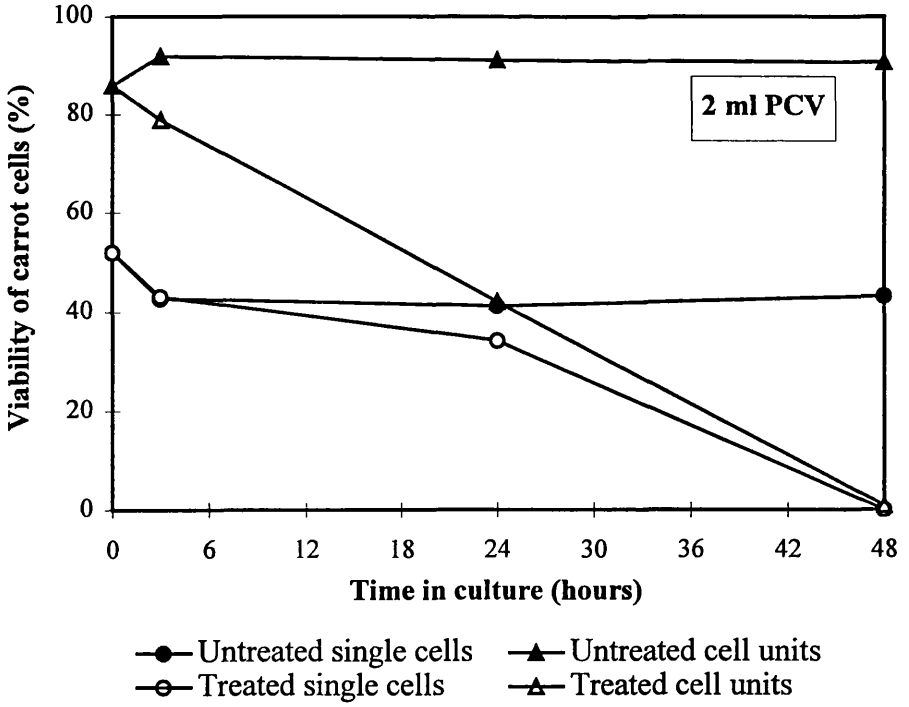


Plate 32

(A)


100
80
60
40
20
0
Viability of carrot cells (%)
1 ml PCV
0
6
12
18
24
30
36
42
48
Time in culture (hours)
Untreated single cells
Untreated cell units
Treated single cells
Treated cell units

(B)


100
80
60
40
20
0
Viability of carrot cells (%)
2 ml PCV
0
6
12
18
24
30
36
42
48
Time in culture (hours)
Untreated single cells
Untreated cell units
Treated single cells
Treated cell units

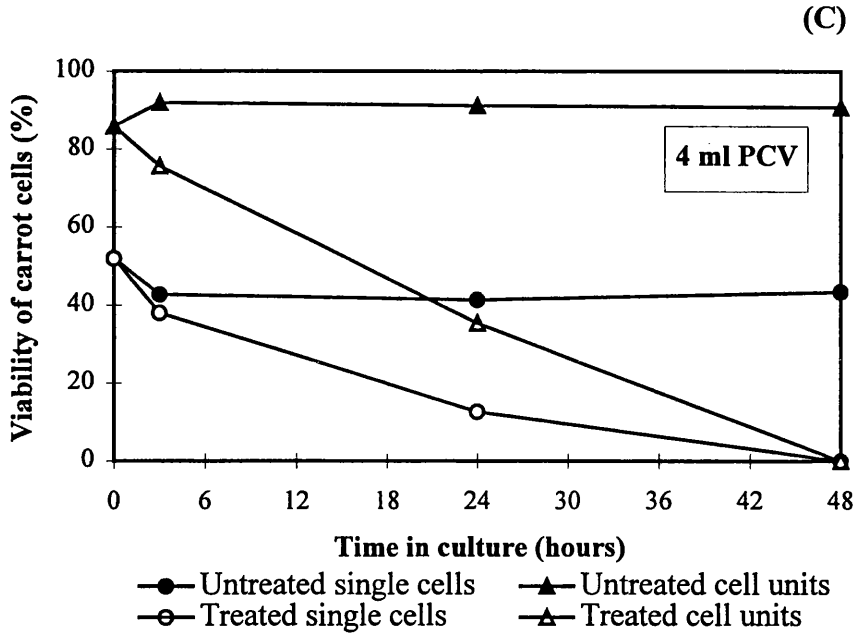


Figure 3.18: Cell viability of carrot cv. Morot Duke suspension culture inoculated with 1, 2 and 4 ml PCV of *P. violae*

4. DISCUSSION

Co-culture and subsequent regeneration of survivors may be a useful tool in developing new crop varieties with enhanced disease resistance.

A successful co-culture technique was developed to study the host pathogen interaction and, with a more suitable antibiotic regime, this technique should provide regenerants which may possess novel disease resistance or tolerance from surviving embryogenic units after challenge by *E. carotovora.*

Co-culture of carrot cells with *E. carotovora* was examined for the effect of medium on survival of both carrot cells and bacteria, for the effect of age of carrot cells on the interaction and for the effect of concentration of bacteria used. Of those tested, MS medium without hormones and sucrose, diluted 1/10 th was considered to be

preferable since it maintained the viability of both bacterial and plant cells without allowing proliferation of bacteria. Furthermore, as this medium does not contain a carbon source, it is tenable that killing of plant cells occurred as a result of toxic factor (s) resulting from the host pathogen interaction rather than spurious culture metabolites. This medium was also reported to resemble the dilute nutrient conditions which would be found in the intercellular species in leaves (Matthysse, 1987). 1/10 th MS medium was found to be suitable to expose a hypersensitive-like response in suspension cultures of carrot and tobacco when inoculated with the incompatible bacterium *Pseudomonas syringae* pv. *phaseolicola* (Matthysse, 1987).

Cells in log phase (8-day) were selected as the most suitable for subsequent experiments since the viability of plant cells did not decrease in 1/10 th control medium and also a proportion of plant cells in co-culture experiments remained viable up to 48 hrs. Although there has been no published report on the relationship between age of plant cells *in vitro* and cell killing when inoculated with bacteria, plant cells at the exponential stage have often been used for co-culture experiments in many bacterial host interactions (Atkinson et al., 1985; Matthysse, 1987; Roach and Garnett, 1986).

Co-culture of carrot cells with pathogenic *E. carotovora* resulted in reduced the viability of carrot single cells and cell units. Increasing concentrations of bacteria resulted in decreasing viability of plant cells in suspensions. Killing of carrot cells first became evident by 2 hrs after inoculation when the survival of cell units was reduced by 7% at 10^3 cfu/ml and 20% at 10^9 cfu/ml; over 85% carrot cells was killed within 24 hrs. Similar results were observed by Roach and Garnett (1986) when cassava cells were inoculated with pathogenic strains of *Erwinia herbicola* at 10^7 cfu/ml. The population of *E. herbicola* increased 100 fold by 72 hrs by which time all cassava cells had been killed.

Optimization of the inoculum concentration has also been shown to be important in *in vitro* expression of disease resistance. The differences between calli from resistant and susceptible potato genotypes tended to blur at high *P. infestans* inoculum levels and careful control of inoculum levels was found to be necessary for the expression *in vitro* of resistance against *Didymella applanata* in raspberry callus cultures (Czech-Kozlowska and Krzywanski, 1984).

Mutants defective in secretion of extracellular enzymes were used in an attempt to investigate the factors responsible for pathogenicity by comparing virulence with the wild type strains. The multiplication rate of wild type SCRI 193 and one of the enzyme deficient mutants RJP 243 was 20 times greater in co-cultures than in 1/10 th MS medium in which there was a 2-fold increase in 24 hrs. By contrast, enzyme deficient mutant RJP 116 increased only 3-fold in co-culture but decreased in 1/10 th MS medium. In general, multiplication of the enzyme deficient mutants increased in co-cultures but to a lesser extent than the very rapid increase with wild type SCRI 193. Fifty and thirty times increase by 24 hrs in bacterial numbers of pathogenic and non-pathogenic strains of *E. herbicola* respectively occurred when co-cultured with cassava cells according to Roach and Garnett (1986). Difference in the extent of increase of the bacterial population may indicate the events of the host-parasite interaction. As there was no carbohydrate source in the 1/10 th MS medium, *Ecc* must have utilizing carbohydrates from degraded carrot cells.

Killing of plant cells by soft rot bacteria is a phenomenon widely accepted as due to cell wall degrading enzymes. Most evidence indicates that PGL and/or PL are responsible for killing of plant tissues, although other enzymes such as endo-PG can also do this (Basham and Bateman, 1975a, b; Mount et al., 1970). The very rapid and extensive killing by wild type *Ecc*, is probably caused by extracellular "toxic" pectate

lyase. This conclusion is based on: i) the enzyme was detected in culture fluids derived from co-culture treatments, axenic culture *in vitro* and also in *E. carotovora* infected carrot tissues *in vivo*; ii) there was a close correlation between the rate of cell killing, increase in bacterial number and also extracellular PGL activity in co-culture treatments; iii) the pathogenic strain of *Ecc* (wild type SCRI 193) killed plant cells faster and to higher levels compared to the two enzyme deficient mutants RJP 243 and RJP 116. During the first 24 hrs when RJP 116 failed to cause a marked decrease in the viability of carrot cells in 24 hrs, no PGL activity was detected. Wild type *Ecc* SCRI 193 and over enzyme producing mutants were much more pathogenic to carrot taproots than enzyme deficient mutants as seen in Section 2.2.1.

However, interpretation of these data solely in terms of PGL must be made with caution because the Warwick mutants of *Ecc* were pleiotropically defective. The global regulatory loci coordinately controlled the synthesis of several extracellular cell wall degrading enzymes (pectate lyase, cellulase, protease, polygalacturonases) (Salmond, 1994). Single gene disrupted mutants of *E. chrysanthemi* were available and in structural genes for PGL. It was unfortunate therefore that *E. chrysanthemi* mutants proved to be non-pathogenic to carrot roots (Boccara et al., 1988).

The cell killing of suspension cultured carrot cells (over 90%) by pathogenic *E. carotovora* (SCRI 193 at 10^7 cfu/ml) during the first 24 hrs was faster than that of reported for the killing of cassava cells (40% in 24 hrs) by *E. herbicola* (Roach and Garnett, 1986). The effect of *E. carotovora* and *E. atroseptica* on carrot callus tissues was investigated by Volcani et al. (1953) who revealed that carrot callus tissues discoulored in 3 days but no typical soft rot was observed on calli when inoculated with *E. carotovora* whereas soft rot was evident with *E. atroseptica* on the carrot calluses. Tobacco isolates of *Ecc* caused necrosis of inoculated tobacco tissue cultures in 24 hrs

at low concentrations (10 bacteria per callus) while non-tobacco isolates of *Ecc* caused callus maceration 2 days after inoculation (Mc Intyre et al., 1978). Roach and Garnett (1986) illustrated that cell suspension cultures of two cassava cultivars showed almost complete cell killing in 3 days when inoculated with two pathogenic strains of *Erwinia herbicola*. However, the killing of the cassava plant cells was highly reduced when non-pathogenic strains were used. Taylor and Secor (1985; 1990) also illustrated that 90% of protoplast derived potato calli were killed within 5 days when calli were exposed to *E. carotovora* pv. *carotovora* (*Ecc* 71).

Davis et al. (1984) showed that endopolygalacturonic acid lyase purified from *E. carotovora* triggered the elicitation of phytoalexin accumulation by releasing oligosaccharides from the pectic polymers of soybean (*Glycine max* L.) cotyledon cell walls. Amin et al. (1986) also reported that extracellular pectinolytic enzymes of various fungi (*Chaetomium globosum*, *Botrytis cinerea*, *Fusarium moniliforme*, *Helminthosporium oryzae*) elicited the phytoalexin, 6-methoxymellein, accumulation in carrot suspension culture. It could be possible that high PGL produced by wild type SCRI 193 may kill carrot cells very rapidly and thereby might prevent carrot cells from mounting a successful defense mechanism. High PGL activity may also degrade the endogenous elicitors which serve as regulatory molecules that initiate the synthesis and accumulation of phytoalexins in infected tissues. Therefore, carrot cells were not able to accumulate phytoalexins in co-culture at effective levels. However, PGL$^-$ mutants may have triggered phytoalexin accumulation.

Rapid cell killing coincided with appearance of extracellular PGL in many experiments. It can be suggested that the aggressiveness of *E. carotovora* at the cellular level is mainly due to the secretion of PGL into the culture medium. Basham and Bateman (1975a; b) reported that purified PGL from *E. chrysanthemi* caused both tissue

maceration and cell death in potato and tobacco tissues involving rapid release of unsaturated uronides, ions and water from cytoplasm (*ca.* 50% in 1 hour) of intact plant tissues. Other enzymes could be associated with cellular death of plant tissues as shown by Tseng and Mount (1974). Highly purified protease and phosphatidase C produced by *E. carotovora* caused cellular death while purified endo-PG caused lysis of cucumber protoplasts. These findings suggested that the cell membranes of cucumber protoplast were damaged by protease and phosphatidase of *E. carotovora* while endo-PG had no effect on the membranes.

The "Missouri" mutants of *Ecc* were either less pathogenic or non-pathogenic to carrot roots *in vivo* and, coincidentally, (see Section 2.2.2 and 2.6.1.2) this *E. carotovora* strain and mutants caused less effect on carrot cells compared to the Warwick mutants in suspension cultures. Once again, it was confirmed that when enzyme deficient mutants were compared with wild type strains, they were associated with limited or no production of PGL *in vivo* and *in vitro*. Somewhat analogously, Beraha and Garber (1971) reported that avirulent mutants of *E. carotovora* had markedly less pectinesterase, polygalacturonase, pectate lyase and cellulase than three virulent strains *in vitro* on media containing polygalacturonic acid and carboxymethylcellulose. The growth of protoplast derived potato calli on media containing culture filtrates of *Ecc* was inhibited after 28 days exposure to culture filtrates, which was obtained from *Ecc* grown in a minimum salts medium; complete cell death after 70 days correlated with pectinase activity (Taylor and Secor, 1987). Purified pectin lyase (PL) from *Monilinia fructigena* caused death of apple suspension culture, whereby a higher PL concentration (6.8 units/ml) caused the death of almost all cells within 35 minutes while lower PL (2.5 units/ml) killed 65% of the cells (Hislop et al., 1979). Mount et al. (1970) reported the toxic effect of PGL from *E. carotovora* on

potato discs linked with changing permeability, maceration and cellular death of potato tissues.

Although PGL appears to contribute to death of carrot cells, killing by wild type SCRI 193 first became evident by 2 hrs after inoculation when survival was reduced by 40% at 10^7 cfu/ml. There was no detectable PGL by that time. It could be possible that factors other than PGL initiated cell killing first; subsequent accumulation of PGL, host phenolic compounds, enzymes and electrolytes released from moribund cells in the culture medium might speed the killing of other viable carrot cells (Mount et al., 1970). Stephens and Wood (1975) also illustrated that PGL killed the unplasmolysed protoplasts of carrot cells but failed to kill plasmolysed protoplasts. They suggested that some factor(s) other than PGL killed the plasmolysed carrot tissues.

Sterile culture fluids containing PGL taken from either axenic culture or the interaction between carrot cells and *E. carotovora* (wild type SCRI 193) were highly toxic to carrot cell suspension cultures. Pectate lyase at 0.47 μkat caused the death of the majority of cells within 2 hrs (80%) when applied to suspension cultures of carrot while lower PGL activity (0.0028 μkat) did not affect the viability of carrot cells. However, culture fluids from enzyme deficient mutant RJP 116 (containing 0.11 μkat PGL) did not reduce the viability at all. Crude culture fluids of *E. carotovora* may contain other toxic components or other enzymes which assist either direct killing of carrot cells or indirectly by inducing the cell wall damage in the carrot cells. It is tenable that failure to detect them was because other "toxins" were cell-bound; their identification was beyond the scope of this study.

Selection of plant cells for resistance to crude culture fluids of pathogens *in vitro* has been successfully studied by various workers to select resistant plants (Behnke, 1979; Binarová et al., 1990; Hammerschlag et al., 1994). Binarová et al. (1990) using

highly embryogenic suspension cultures of alfalfa to select resistant plants from culture filtrates of *Fusarium* spp., obtained 12-20% of the 167 regenerated alfalfa plants with increased resistance to the pathogen. MacDonald and Ingram (1986) selected stable resistant plants from secondary embryoids of oilseed rape (*Brassica napus*) exposed to partially purified culture filtrates of *Alternaria brassicicola*. Increased resistant peach (*Prunus persica* L.) regenerants were selected for insensitivity to the crude culture filtrates of bacterial leaf spot (*Xanthomonas campestris* pv. *pruni*) by Hammerschlag et al. (1994). When resistant plants were tested in the greenhouse and under field conditions, resistance was stable at the whole plant level. Selection *in vitro* for novel resistance to putative phytotoxic compounds or crude culture filtrates has not always resulted in successful selection. Newsholme et al. (1989) failed to select resistant winter oilseed rape (*Brassica napus* ssp. *oleifera* [Metzg.] Sinsk.) plants from secondary embryogenic cultures to phytotoxic products of *Leptosphaeria maculans*.

In addition, co-culture of carrot cells with incompatible bacteria or fungi could be a suitable system to study the biochemical mechanisms associated with the hypersensitive reactions *in vitro*. The hypersensitive reaction (HR) is known to involve the rapid death of plant cells as a response to incompatible pathogens (Duvick and Sequeira, 1984). Atkinson et al. (1985) demonstrated HR to *Pseudomonas syringae* pv. *pisi* (incompatible with tobacco) of suspension-cultured-tobacco cells. Tobacco cells responded to incompatible bacteria by electrolyte loss, decreased respiration rate, brown pigmentation and finally cell death in 1.5 hrs after inoculation.

Bacterial attachment to plant cell surfaces may be an initial step in a series of events causing carrot cell killing. Duvick and Sequeira (1984) demonstrated that an avirulent strain of *Pseudomonas solanacearum* induced hypersensitive-like reaction on suspension-cultured-tobacco cells. The avirulent strain attached more rapidly to tobacco

cells and to leaf cell walls than the virulent strain. This could be the case for *E. carotovora*, whereby virulent SCRI 193 may remain free and may increase their population in the intercellular spaces of the carrot tissues.

Co-culture of *Ecc* and carrot cell suspension cultures should be a suitable system for determining if contact between the plant cells and bacterial cells was essential for the death of carrot cells. Youle and Cooper (1987) showed that when apple suspension cells were co-incubated with *E. amylovora* cells and separated by dialysis tubing, cell killing did not occur.

Antibiotics were used here to remove bacteria from embryogenic units challenged in co-culture without damaging the carrot cells. Several antibiotics other than rifampicin and cycloheximide were effective in removing *Ecc* and did not significantly reduce the viability of carrot cells up to 3 days. Nevertheless, a key factor was the subsequent proliferation and regeneration of selected carrot cells after co-culture followed by antibiotic treatments. Although kanamycin, which was chosen at 25 μg/ml to remove bacteria from co-cultures, did not reduce the viability of carrot cells up to 4 days, it seriously reduced the regeneration capacity of the carrot cells. Dix et al. (1977) found a cell line of *Nicotiana sylvestris* with resistance to kanamycin and streptomycin at 50 μg/ml. Although the resistant line, KR103, proliferated as callus in culture medium on regeneration medium it exhibited no shoot or root formation. Kanamycin and streptomycin are known as aminoglycoside antibiotics, which inhibit protein synthesis by binding "bacterial like" ribosomes (Pestka, 1971). Watts and King (1973) demonstrated that kanamycin at 25 μg/ml affected the protein biosynthesis of *Antirrhinum majus*, *Pisum sativum* and *Nicotiana tabacum* by reducing the incorporation of amino acids into proteins by 60%. Therefore, there was no division of protoplasts although protoplasts survived for up to 20 days and this concentration also

gave moderate control of bacterial growth. It is possible that kanamycin also affected division of carrot cells. Kanamycin, is a widely used selection agent for plant transformations. Kanamycin has been reported to inhibit completely *in vitro* shoot organogenesis in *Rubus* (raspberry and blackberry) at 10 mg/ml when used to screen for transformation (Fiola et al., 1990). When using kanamycin to select and regenerate for transgenic plants, Yepes and Aldwinckle (1994) also found that kanamycin strongly inhibited regeneration efficiency in apple even when used at very low doses. The toxic effect of kanamycin to *Nicotiana plumbaginifolia* cells at 10 μg/ml was also shown by Pollock et al. (1983). In contrast, kanamycin, when added to a shoot-inducing medium at low levels (5-20 μg/ml) has been shown to promote morphogenesis in tobacco and carrot cells (Owens, 1979). Obviously the use of an alternative antibiotics without deleterious effects on carrot cells is required to remove *Ecc* from co-cultures.

Co-cultures of carrot cells and small mycelial fragments of *P. violae* was developed to study the host-pathogen interaction. To date, no report has been published on pathogenicity of *Pythium* spp. *in vitro*. However, carrot cells were highly sensitive to *P. violae* in co-culture, thus 60% of the carrot cells were killed in 24 hrs. This very rapid effect may be a true reflection of the potential pathogenicity of *P. violae*, although it does not fit with the slow, limited invasion achieved in carrot roots. Perhaps the major constraint *in planta* is imposed by the suberized periderm, along with the limited saprotrophic ability of *P. violae.* However, manipulation of the conditions used in co-culture study could be altered to achieve a more realistic, balanced host-pathogen interaction. Such an *in vitro* system could then possibly be used to investigate pathogenicity factors of this poorly understood parasite. Moreover, with modification the method might be valuable as a selection pressure for obtaining carrot regenerants *in vitro* against *P. violae*.

CHAPTER 4

GENERAL DISCUSSION AND FUTURE WORK

One of the major purposes of this study was achieved by the establishment of co-culture techniques between carrot cells and *E. carotovora* or *P. violae*. This is the first report to date concerning studies *in vitro* of these host-parasite combinations.

Highly embryogenic callus and suspension cultures of carrot provide an excellent model system for the study of host-parasite interaction and for selection of disease resistant plants using either pathogens or their pathogenicity determinant(s). In particular co-culture should be suitable for various bacterial pathogens as well as *Ecc*, because rapid and intimate pathogen-host cell contact can be achieved.

Transmission electron microscopy should be used to study the early events of interactions between *E. carotovora* and *P. violae* and carrot cells; the host material, as dispersed, small, non-lignified cell units, are ideal for the stages of fixation and embedding and the time of the interaction can be precisely defined.

Although the co-culture technique is relatively simple and rapid to set up, it is still necessary to screen large numbers of regenerants for selection of desirable plants with the associated time and space required for conventional screening. Whilst screening plants is facile for diseases such as powdery mildews, cavity spot presents some problems because of the lengthy and rather inefficient inoculation procedure.

Although barley seeds infested with *P. violae* increased the diameter of cavity spot formation on carrot roots, the incidence of disease was still erratic as previously found with V8 agar inoculum. 25-30% of the inoculum was unsuccessful in a producing lesion; however multiple inoculations of single roots largely overcame this problem. Future work should concentrate on developing a more effective inoculation technique.

"Toxins" (or other microbial products) should, ideally, only be used when they have been shown to be clearly involved in disease. There are relatively few examples of these; thus use of intact pathogen cells must be preferable as for the two facultative pathogens of carrot here, but as long as pathogenicity factors are expressed under *in vitro* conditions. The rapid killing of carrot cells by *Ecc* generally coincided with production of extracellular PGL, which gave some confidence in the methodology, as did the slower, reduced cell killing by low pathogenicity mutants.

Disease resistance based on the hypersensitive response is thought to be based on recognition phenomena, which is likely to involve the cell surface of the pathogen (Callow, 1987; Mazzucchi, 1983). The enzyme deficient mutants e.g. RJP 116 and RJP 243 could therefore be useful in selection for resistant lines to *E. carotovora* because they lack "toxic" PGL but should fully retain surface characteristics. PGL kills cells of most plant species rapidly (Bateman and Basham, 1975; Cooper, 1983); thus it is difficult to envisage disease resistance of a cultivar based on resistance to PGL and a selection system may be more likely to succeed in the absence of this enzyme.

Nevertheless, if toxic factors other than PGL are produced by *Ecc* (there was some evidence for this from co-culture experiments) it is tenable that they could be used in *in vitro* selection.

There is little information on the pathogenicity of *P. violae* on carrot roots but in the current study, it was shown that *P. violae* secretes the cell wall degrading enzymes, PL, cellulase and suberinase, which presumably facilitate penetration of unwounded periderm of carrot roots. Future work should focus on the determination of whether pathogen produced phytotoxins (in view of the very rapid killing of suspension cells) or enzymes are involved in pathogenicity. A phytotoxin from *P. violae* could offer a useful selection agent *in vitro*.

Regeneration was attempted from *ca.* 30% of carrot cells which remained viable after challenging with bacteria; allowing viability to be reduced to 10% or less should increase the chance of obtaining possible resistant mutants from co-cultured carrot cells, but the likelihood of obtaining sufficient numbers of regenerants will be reduced.

In spite of the establishment of a co-culture technique, a few technical problems arose. Although, plant cells were successfully challenged with *E. carotovora* and bacteria were removed from co-cultured plant cells by kanamycin in 24 hrs, kanamycin affected the regeneration capacity of carrot cells at the concentrations used. Thus it was not possible to select resistant cell lines from co-culture treatments. It remains to find a suitable antibiotic to perform the final stage of this new technology.

In this study, scanning electron microscopy showed no obvious differences between resistant and susceptible lines of carrot to powdery mildew in terms of the fungal development on leaves. It remains to determine where and how the development of *E. heraclei* is halted in the resistant, regenerated Somaclone-4. The biochemistry of antimicrobial compounds and other defence responses in carrot have already been

described (Chakravarty and Srivastava, 1967; Davies and Lewis, 1981; Kurosaki and Nishi, 1984) but never in relation to resistance to powdery mildew or to *P. violae*.

Cavity spot lesions on the roots remained superficial and limited (2-4 mm deep, 2-15 mm in length) (Guba et al., 1961; Perry and Harrison, 1979a). This suggests that carrot roots react to *P. violae* attack to restrict infection. Chakravarty and Srivastava (1967) reported that inhibition of the growth of *Pythium aphanidermatum* in carrot roots was related to phenolic compounds in roots. Detailed investigation of the interaction with *P. violae* should be conducted.

Effective resistance to *E. heraclei* clearly exists in seed populations and should be incorporated into a breeding programme. Other carrot genotypes reflecting all the main commercial types should be screened to reveal whether resistance is widespread in the species.

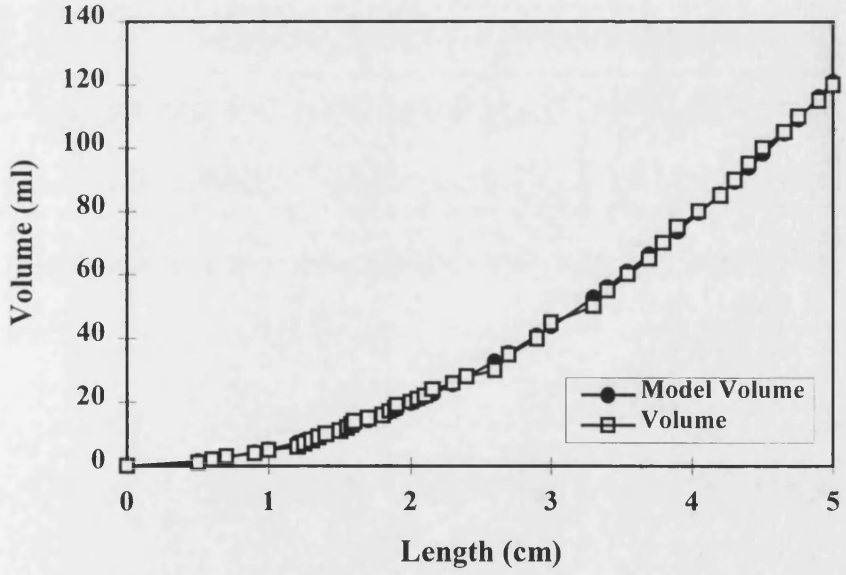
Information on the genetics of resistance of carrot to *E. heraclei* would also provide a better understanding of the nature of disease resistance and aid in its optimal use under field conditions.

The general absence of resistance to soft rot bacteria in cultivars of most species and of carrot to *P. violae* further encourages the continued development of novel resistance *via* somaclonal variation, perhaps to succeed where conventional hybridisation breeding has failed.

APPENDICES

I. ANALYSIS OF VARIANCE

APPENDIX 1: The correlation between cell volume obtained from experimental results and cell volume obtained from model volume formula



Model volume = 0.313128 + (–0.04798) × L + 4.836076 × (L × L)
L=length
n=45 (including 0)

Legend for Appendices where applicable

***	significant, $p < 0.01$
*	significant, $p < 0.05$

APPENDIX 2: The effects of length of hypocotyl, site on cotyledon, light, wounding of cotyledon explant and media on somatic embryo production with cotyledon explants from carrot cv. NRI-92

ANALYSIS OF VARIANCE (Based on data from Chap. 3.I, Sec. 2.1.1., Table 3.1)

SOURCE	df	SS	MS	F	F_{table}	
					0.05	0.01
Media	1	0.142	0.142	2.19	4.21	7.68
Wounding	1	2.038	2.038	31.45***	4.21	7.68
Light	1	0.665	0.665	10.26***	4.21	7.68
Site	1	3.614	3.614	55.27***	4.21	7.68
Size	2	8.874	4.437	68.47***	3.35	5.49
Media × Wounding	1	0.003	0.003	0.04	4.21	7.68
Media × Light	1	0.251	0.251	3.87	4.21	7.68
Media × Size	2	0.314	0.157	2.42	3.35	5.49
Media × Site	1	0.186	0.186	2.87	4.21	7.68
Wounding × Light	1	0.021	0.021	0.032	4.21	7.68
Wounding × Site	1	0.121	0.121	1.86	4.21	7.68
Wounding × Size	2	0.053	0.026	0.40	3.35	5.49
Light × Site	1	0.155	0.155	2.39	4.21	7.68
Light × Size	2	0.255	0.127	1.95	3.35	5.49
Site × Size	2	0.151	0.076			
ERROR	27	1.75	0.065			
TOTAL	47	18.593				

APPENDIX 3: The effects of the seedling age (A), wounding (B), seedlings light treatment (C), site on cotyledon (D) on somatic embryogenesis of cotyledon explant

Re-arranged data from Chap. 3.I, Sec. 2.1.1.

(A)

Length of hypocotyl (mm)	Embryogenic response (%)	Average somatic embryo* production
1-3	70.1	1.5 a
4-6	50.0	0.7 b
8-10	35.1	0.5 c

(B)

Wounding	Embryogenic response (%)	Average somatic embryo* production
Wounded	59.3	1.2 a
Intact	43.7	0.7 b

(C)

Condition of Light	Embryogenic response (%)	Average somatic embryo* production
Dark	57.0	1.0 a
Light	46.1	0.8 b

(D)

Type of cotyledon explant	Embryogenic response (%)	Average somatic embryo* production
Base	65.0	1.2 a
Top	38.0	0.6 b

*: scoring system (see Section 2.1). Means within columns followed by the same letters are not significantly different according to Duncan's Multiple Range Test at 5% level. Means were calculated from 12 samples for treatment A and that was 24 for treatments B, C, D. The light/dark related to treatments with the seedlings.

APPENDIX 4: The effects of length of hypocotyl, light and media on somatic embryo production with hypocotyl explants from carrot cv. NRI-92

ANALYSIS OF VARIANCE (Based on data from Chap. 3.I, Sec. 2.1.2., Table 3.2)

SOURCE	df	SS	MS	F	F_{table} 0.05	0.01
Media	1	0.414	0.414	3.55	18.51	98.50
Light	1	0.011	0.011	0.09	18.51	98.50
Size	2	2.104	1.052	9.04	19.00	99.00
Media × Light	1	0.017	0.017	0.146	18.51	98.50
Media × Size	2	0.640	0.320	2.75	19.00	99.00
Light × Size	2	0.080	0.080	0.343	19.00	99.00
ERROR	2	0.233	0.116			
TOTAL	11	3.499				

APPENDIX 5: The effects of explant, concentration of 2,4-D and basal media on somatic embryo production from carrot cv. NRI-92

ANALYSIS OF VARIANCE (Based on data from Chap. 3.I, Sec. 2.2., Table 3.3)

SOURCE	df	SS	MS	F	F_{table} 0.05	0.01
Media	1	0.154	0.154	0.383	10.13	34.12
Explant	1	0.007	0.007	0.017	216	5,403
Concentrations	3	0.511	0.170	0.424	9.28	29.46
Media × Explant	1	0.001	0.001	0.003	216	5,403
Media × Concent.	3	0.306	0.102	0.253	9.28	29.46
Explant × Concent.	3	0.239	0.080	0.199	9.28	29.46
ERROR	3	0.402	0.134			
TOTAL	15	1.619				

APPENDIX 6: The effects of concentration of 2,4-D and time of callus exposure to embryo induction medium on somatic embryo production with hypocotyl explants from carrot cv. NRI-92

ANALYSIS OF VARIANCE (Based on data from Chap. 3.I, Sec. 2.3., Table 3.4)

SOURCE	df	SS	MS	F	F_{table} 0.05	0.01
Stage	2	0.091	0.046	0.289	19.0	99.0
Concentrations	1	0.020	0.020	0.125	200.0	4,999
ERROR	2	0.317	0.159			
TOTAL	5	0.429				

APPENDIX 7: The effects of the type of explant and the concentration of 2,4-D in embryo induction medium on somatic embryo production from four carrot cultivars

ANALYSIS OF VARIANCE (Based on data from Chap. 3.I, Sec. 2.4., Table 3.5)

SOURCE	df	SS	MS	F	F_{table} 0.05	0.01
Cultivar	3	3.088	1.029	25.88***	3.86	6.99
Explant	1	2.290	2.290	57.57***	5.12	10.56
Concentrations	3	4.335	1.445	36.33***	3.86	6.99
Concentrations × Explant	3	1.432	0.477	36.00***	3.86	6.99
Cultivar × Concentrations	9	0.934	0.104	2.62	3.18	5.35
Cultivar × Explant	3	1.518	0.506	12.72***	3.86	6.99
ERROR	9	0.358	0.040			
TOTAL	31	13.955				

APPENDIX 8: The effect of 2,4-D concentrations in embryo induction medium (A) and types of explant (B) on somatic embryogenesis with four carrot cultivars (C)

Re-arranged data from Chap. 3.I, Sec. 2.4.

(A)

2,4-D Concentrations (μM)	Embryogenic response (%)	Average somatic embryo* production
0.05	38.9	0.47 c
0.5	63.9	1.01 b
5	71.0	1.48 a
50	60.1	1.16 b

(B)

Explant	Embryogenic response (%)	Average somatic embryo* production
Hypocotyl	60.3	1.30 a
Cotyledon	56.6	0.76 b

(C)

Cultivars	Embryogenic response (%)	Average somatic embryo* production
Morot Duke	65.0	1.39 a
Morot Favor	74.5	1.30 a
Morot Ingot	50.0	0.76 b
NRI-92	44.5	0.68 b

Means within columns followed by the same letters are not significantly different according to Duncan's Multiple Range Test at 5% level. Means were calculated from 80 replicated samples for treatments A and C and 160 replicated samples for treatments B

APPENDIX 9: The effect of concentration of 2,4-D on mature somatic embryo formation of callus cultures from four carrot cultivars

ANALYSIS OF VARIANCE (Based on data from Chap. 3.I, Sec. 2.5, Table 3.6)

SOURCE	df	SS	MS	F	F_{table} 0.05	0.01
Cultivar	3	476242	158747	5.66***	2.74	4.07
Concentrations	1	11446602	11446602	407.99***	3.98	7.01
Cult. × Concent.	3	881517	293839	10.47***	2.74	4.07
ERROR	72	2020032	28056			
TOTAL	79	14824393				

APPENDIX 10: The effect of embryo initiation media and of suspension culture maintenance media on subsequent growth of carrot cv. Morot Duke-8 and Morot Duke-39 suspension cultures

ANALYSIS OF VARIANCE (Based on data from Chap. 3.I, Sec. 3.4, Figure 3.3)

SOURCE	df	SS	MS	F	F_{table} 0.05	F_{table} 0.01
Lines	1	2.202	0.016	1467.80***	5.32	11.26
Media	1	0.016	0.016	10.75*	5.32	11.26
Line × Media	1	0.059	0.059	39.2***	5.32	11.26
ERROR	8	0.012	0.002			
TOTAL	11	2.286				

APPENDIX 11: The effect of cell unit size and regeneration media on mature somatic embryo production from long term suspension cultures

ANALYSIS OF VARIANCE (Based on data from Chap. 3.I, Sec. 3.5.1, Table 3.8)

SOURCE	df	SS	MS	F	F_{table} 0.05	F_{table} 0.01
Fraction	3	1075	358	22.38***	2.90	4.46
Media	3	4360.7	1453.6	90.85***	2.90	4.46
Fraction × Media	9	1431	159	9.93***	2.19	3.02
ERROR	32	511.4	16			
TOTAL	47	7378				

APPENDIX 12a: Determination of pathogenicity of *E. carotovora* strains 2 days after inoculation

ANALYSIS OF VARIANCE (Based on data from Chap. 3.II, Sec. 2.1, Table 3.9)

SOURCE	df	SS	MS	F	F_{table} 0.05	F_{table} 0.01
Strains	1	16.22	16.22	77.87***	4.11	7.4
Depths	1	3.98	3.98	19.11***	4.11	7.4
Concentrations	2	22.88	11.44	54.94***	3.26	5.25
S × D	1	0.58	0.58	2.77	4.11	7.4
S × C	2	2.14	1.07	5.14*	3.26	5.25
D × C	2	0.27	0.14	0.65	3.26	5.25
S × C × D	2	1.17	0.09	0.41	3.26	5.25
ERROR	36	7.497	0.21			
TOTAL	47	53.734				

APPENDIX 12b: Determination of pathogenicity of *E. carotovora* strains 4 days after inoculation

ANALYSIS OF VARIANCE (Based on data from Chap. 3.II, Sec. 2.1, Table 3.9)

SOURCE	df	SS	MS	F	F_{table} 0.05	0.01
Strains	1	23.77	23.77	59.89***	4.11	7.4
Depths	1	22.85	22.85	57.57***	4.11	7.4
Concent.	2	75.32	37.66	94.89***	3.26	5.25
S × D	1	0.12	0.12	0.30	4.11	7.4
S × C	2	3.73	1.87	4.70*	3.26	5.25
D × C	2	0.43	0.21	0.53	3.26	5.25
S × C × D	2	1.61	0.81	2.03	3.26	5.25
ERROR	36	14.29	0.397			
TOTAL	47	142.13				

APPENDIX 13a: Determination of virulence of different mutants and wild types of *Ecc* obtained from Warwick University at 10^7 and 10^9cfu/ml inoculum concentrations

ANALYSIS OF VARIANCE (Based on data from Chap. 3.II, Sec. 2.2.1, Table 3.10)

SOURCE	df	SS	MS	F	F_{table} 0.05	0.01
Strains	6	64.74	10.79	17.63***	2.85	4.46
Concentrations	1	19.86	19.86	32.45***	4.60	8.86
Interaction	6	1.86	0.309	0.50	2.85	4.46
ERROR	14	8.57	0.612			
TOTAL	27	95.03				

APPENDIX 13b: Determination of virulence of different mutants and wild types of *Ecc* obtained from Missouri University at 10^7 and 10^9 cfu/ml inoculum concentrations

ANALYSIS OF VARIANCE (Based on data from Chap. 3.II, Sec. 2.2.2, Table 3.11)

SOURCE	df	SS	MS	F	F_{table} 0.05	0.01
Concentrations	1	0.29	0.29	0.14	60.71	244.00
Strains	1	64.80	64.8	31.60***	4.75	9.33
Interaction	1	0.09	0.09	0.04	60.71	244.00
ERROR	12	24.59	2.05			
TOTAL	15	89.77				

APPENDIX 13c: Determination of virulence of different mutants and wild types of *Ech* obtained from Cornell University at 10^7 and 10^9 cfu/ml inoculum concentrations

ANALYSIS OF VARIANCE (Based on data from Chap. 3.II, Sec. 2.2.3, Table 3.12)

SOURCE	df	SS	MS	F	F_{table} 0.05	0.01
Strains	5	115.06	23.01	47.14***	2.41	3.43
Concentrations	1	2.57	2.57	5.26***	4.04	7.19
Interaction	5	1.60	0.32	0.65*	2.41	3.43
ERROR	48	23.43	0.49			
TOTAL	59	142.7				

APPENDIX 14: Growth of *E. carotovora* wild type and mutants on carrot roots

ANALYSIS OF VARIANCE (Based on data from Chap. 3.II, Sec. 2.3., Table 3.13)

SOURCE	df	SS	MS	F	F_{table} 0.05	0.01
Strains	3	6275	2091.7	40.69***	4.07	7.59
ERROR	8	411. 2	51.4			
TOTAL	11	6686.2				

*** significant, $p < 0.01$

APPENDIX 15a: Effect of taproot size on the cavity spot formation of carrot roots

Chi-Square Test (Based on data from Chap. 3.II, Sec. 3.1., Table 3.16)

Size	With Cavity		Without Cavity	
	Observed	Expected	Observed	Expected
Small	38.0	35.64	18.0	20.36
Medium	68.0	71.27	44.0	40.73
Large	34.0	33.09	18.0	18.91
Total	140.0			

($\mathbf{X^2} = 0.912$) < ($\mathbf{X^2} = 5.99$ for 0.05 and 7.38 for 0.01)

APPENDIX 15b: Effect of inoculation site on the cavity spot formation of carrot roots

Chi-Square Test (Based on data from Chap. 3.II, Sec. 3.1., Table 3.16)

Size	With Cavity		Without Cavity	
	Observed	Expected	Observed	Expected
Top (1)	55.0	42.97	14.0	25.45
Middle (2)	19.0	25.53	22.0	16.05
Middle (3)	24.0	25.53	17.0	16.05
Bottom (4)	39.0	42.97	30.0	25.45
Total	137.0			

($X^2 = 13.727$) < ($X^2 = 7.81$ for 0.05 and 11.34*** for 0.01)

APPENDIX 16a: The effect of wounding and medium on cavity spot formation (the mean severity of cavities [mm])

ANALYSIS OF VARIANCE (Based on data from Chap. 3.II, Sec. 3.2., Table 3.17)

SOURCE	df	SS	MS	F	F_{table}	
					0.05	0.01
Treatment	1	1.25	1.25	1.52	4.49	16.0
Media	1	42.44	42.44	51.47***	4.49	16.0
Interaction	1	2.34	2.34	2.84	4.49	16.0
ERROR	16	13.19	0.82			
TOTAL	19	59.22				

APPENDIX 16b: The effect of wounding and medium on cavity spot formation (percentage of carrots with cavity spot)

ANALYSIS OF VARIANCE (Based on data from Chap. 3.II, Sec. 3.2., Table 3.17)

SOURCE	df	SS	MS	F	F_{table}	
					0.05	0.01
Wounding	1	4171	41.7	0.16	7.19	7.09
Media	1	63380	63380.0	241.25***	7.19	7.09
Days	2	13227	6614.0	25.18***	5.19	5.08
Wounding × Media	1	116	116.0	0.44	7.19	7.09
Wounding × Days	2	195	97.0	0.37	5.19	5.08
Media × Days	2	1081	541.0	2.06	5.19	5.08
Wound × Med × Days	2	1676	838.0	3.19	5.19	5.08
ERROR	48	12611	262.7			
TOTAL	59	96457				

APPENDIX 17: The effect of different inoculum types on severity of cavity spot formation of carrot roots

ANALYSIS OF VARIANCE (Based on data from Chap. 3.II, Sec. 3.4.2. and Table 3.20)

SOURCE	df	SS	MS	F	F_{table} 0.05	0.01
Treatments	3	33.80	11.27	3.81*	2.61	5.95
ERROR	12	35.47	2.96			
TOTAL	15	69.27				

APPENDIX 18: The effect of planting time on carrot cv. Morot Duke cavity spot formation

ANALYSIS OF VARIANCE (Based on data from Chap. 3.II, Sec. 3.5., Table 3.21)

SOURCE	df	SS	MS	F	F_{table} 0.05	0.01
Months	3	2.44	0.81	0.27	8.69	26.83
ERROR	16	48.31	3.02			
TOTAL	19	50.76				

APPENDIX 19: Growth of *P. violae* in different carbon sources

ANALYSIS OF VARIANCE (Based on data from Chap. 3.II, Sec. 3.7.2.1., Table 3.24)

SOURCE	df	SS	MS	F	F_{table} 0.05	0.01
Treatments	2	2.647	1.323	28.22***	4.46	8.65
ERROR	6	0.281	0.047			
TOTAL	8	2.928				

APPENDIX 20a: Determination of pathogenicity of *E. heraclei* on regenerated, susceptible and resistant seeds (disease incidence %)

ANALYSIS OF VARIANCE (Based on data from Chap. 3.II, Sec. 4.1., Table 3.31)

SOURCE	df	SS	MS	F	F_{table} 0.05	0.01
Treatments	7	5664.7	809.2	25.85***	3.5	6.18
ERROR	8	250.0	31.3			
TOTAL	15	5914.7				

APPENDIX 20b: Determination of pathogenicity of *E. heraclei* on regenerated, susceptible and resistant seeds (disease severity score)

ANALYSIS OF VARIANCE (Based on data from Chap. 3.II, Sec. 4.1., Table 3.31)

SOURCE	df	SS	MS	F	F_{table}	
					0.05	0.01
Treatments	7	7.28	1.04	4.86*	3.5	6.18
ERROR	8	1.71	0.214			
TOTAL	15	5914.7				

APPENDIX 21a: The level of *E. heraclei* in mechanically inoculated susceptible NRI-92, resistant Somaclone-4 and highly susceptible Danro carrot plants (disease incidence %)

ANALYSIS OF VARIANCE (Based on data from Chap. 3.II, Sec. 4.2., Table 3.32)

SOURCE	df	SS	MS	F	F_{table}	
					0.05	0.01
Treatments	2	7354.4	3677.2	63.96***	4.26	8.02
ERROR	9	517.4	57.5			
TOTAL	11	7871.8				

APPENDIX 21b: The level of *E. heraclei* in mechanically inoculated susceptible NRI-92, resistant Somaclone-4 and highly susceptible Danro carrot plants (disease severity score)

ANALYSIS OF VARIANCE (Based on data from Chap. 3.II, Sec. 4.2., Table 3.32)

SOURCE	df	SS	MS	F	F_{table}	
					0.05	0.01
Treatments	2	10.298	5.149	10.97***	4.26	8.02
ERROR	9	4.223	0.469			
TOTAL	11	14.521				

APPENDIX 21c: The level of *E. heraclei* in naturally inoculated susceptible NRI-92, resistant Somaclone-4 and highly susceptible Danro carrot plants (disease incidence %)

ANALYSIS OF VARIANCE (Based on data from Chap. 3.II, Sec. 4.2., Table 3.32)

SOURCE	df	SS	MS	F	F_{table}	
					0.05	0.01
Treatments	2	12929.4	6464.7	238.34***	4.26	8.02
ERROR	9	244.1	27.1			
TOTAL	11	13173.5				

APPENDIX 21d: The level of *E. heraclei* in naturally inoculated susceptible NRI-92, resistant Somaclone-4 and highly susceptible Danro carrot plants (disease severity score)

ANALYSIS OF VARIANCE (Based on data from Chap. 3.II, Sec. 4.2., Table 3.32)

SOURCE	df	SS	MS	F	F_{table} 0.05	0.01
Treatments	2	21.687	10.843	20.93***	4.26	8.02
ERROR	9	0.807	0.0897			
TOTAL	11	22.494				

APPENDIX 22a: Survival of carrot single cells in different media

ANALYSIS OF VARIANCE (Based on data from Chap. 3.III, Sec. 2.1.1., Figure 3.6a)

SOURCE	df	SS	MS	F	F_{table} 0.05	0.01
Time	6	11935.6	1989.3	30.05***	2.37	2.39
Treatments	4	3870.4	967.6	14.62***	2.64	3.93
Interaction	24	9916.1	413.2	6.24***	1.83	2.46
ERROR	35	2317.2	66.2			
TOTAL	69	28039.2				

APPENDIX 22b: Survival of carrot cell units in different media

ANALYSIS OF VARIANCE (Based on data from Chap. 3.III, Sec. 2.1.1., Figure 3.6b)

SOURCE	df	SS	MS	F	F_{table} 0.05	0.01
Time	6	15174.4	2529.1	25.88***	2.37	2.39
Treatments	4	15668.3	3917.1	40.08***	2.64	3.93
Interaction	24	15525	646.9	6.62***	1.83	2.46
ERROR	35	3420.9	97.7			
TOTAL	69	49788.7				

APPENDIX 23a: Survival of bacterial strain *Ecc* SCRI 1039 suspended in various solutions

ANALYSIS OF VARIANCE (Based on data from Chap. 3.III, Sec. 2.1.2., Figure 3.7a)

SOURCE	df	SS	MS	F	F_{table} 0.05	0.01
Time	6	180.4	30.06	86.74***	2.37	2.39
Treatments	4	102.0	25.50	73.58***	2.64	3.93
Interaction	24	134.3	5.59	16.14***	1.83	2.46
ERROR	35	12.13	0.35			
TOTAL	69	428.84				

APPENDIX 23b: Survival of bacterial strain *Eca* SCRI 139 suspended in various solutions

ANALYSIS OF VARIANCE (Based on data from Chap. 3.III, Sec. 2.1.2., Figure 3.7b)

SOURCE	df	SS	MS	F	F_{table} 0.05	0.01
Time	6	224.7	37.44	24.11***	2.45	3.53
Treatments	3	703.5	234.51	151.02***	2.95	4.57
Interaction	18	206.4	11.47	7.39***	1.99	2.65
ERROR	28	43.5	1.55			
TOTAL	55	1178.1				

APPENDIX 24a: Cell viability of single cells from 2-day-old suspension cultured cells of cultivar Morot Duke inoculated with *Ecc*

ANALYSIS OF VARIANCE (Based on data from Chap. 3.III, Sec. 2.2., Figure 3.8a)

SOURCE	df	SS	MS	F	F_{table} 0.05	0.01
Time	3	9617.12	3205.71	119.73***	4.07	7.59
Treatments	1	69.85	69.85	2.61	5.32	11.26
Interaction	3	170.03	56.68	2.12	4.07	7.59
ERROR	8	214.19	26.77			
TOTAL	15	10071.2				

APPENDIX 24b: Cell viability of single cells from 8-day-old suspension cultured cells of cultivar Morot Duke inoculated with *Ecc*

ANALYSIS OF VARIANCE (Based on data from Chap. 3.III, Sec. 2.2., Figure 3.8a)

SOURCE	df	SS	MS	F	F_{table} 0.05	0.01
Time	3	9063.60	3021.20	19.92***	4.07	7.59
Treatments	1	3015.58	3015.58	19.89***	5.32	11.26
Interaction	3	1063.60	354.53	2.34	4.07	7.59
ERROR	8	1213.08	151.64			
TOTAL	15	14355.9				

APPENDIX 24c: Cell viability of single cells from 14-day-old suspension cultured cells of cultivar Morot Duke inoculated with *Ecc*

ANALYSIS OF VARIANCE (Based on data from Chap. 3.III, Sec. 2.2., Figure 3.8a)

SOURCE	df	SS	MS	F	F_{table} 0.05	0.01
Time	3	9143.09	3047.70	29.84***	4.07	7.59
Treatments	1	1549.77	1549.77	15.17***	5.32	11.26
Interaction	3	937.63	312.54	3.06	4.07	7.59
ERROR	8	817.15	102.14			
TOTAL	15	12447.6				

APPENDIX 24d: Cell viability of cell units from 2-day-old suspension cultured cells of cultivar Morot Duke inoculated with *Ecc*

ANALYSIS OF VARIANCE (Based on data from Chap. 3.III, Sec. 2.2., Figure 3.8b)

SOURCE	df	SS	MS	F	F_{table} 0.05	0.01
Time	3	8507.24	2835.75	48.08***	4.07	7.59
Treatments	1	2376.86	2376.86	40.30***	5.32	11.26
Interaction	3	3498.00	1166.00	19.77***	4.07	7.59
ERROR	8	471.83	58.98			
TOTAL	15	14853.9				

APPENDIX 24e: Cell viability of cell units from 8-day-old suspension cultured cells of cultivar Morot Duke inoculated with *Ecc*

ANALYSIS OF VARIANCE (Based on data from Chap. 3.III, Sec. 2.2., Figure 3.8b)

SOURCE	df	SS	MS	F	F_{table}	
					0.05	0.01
Time	3	10795.69	3598.57	45.61***	4.07	7.59
Treatments	1	3322.24	3322.24	42.11***	5.32	11.26
Interaction	3	3947.07	1315.69	16.68***	4.07	7.59
ERROR	8	631.15	78.89			
TOTAL	15	18696.2				

APPENDIX 24f: Cell viability of cell units from 14-day-old suspension cultured cells of cultivar Morot Duke inoculated with *Ecc*

ANALYSIS OF VARIANCE (Based on data from Chap. 3.III, Sec. 2.2., Figure 3.8b)

SOURCE	df	SS	MS	F	F_{table}	
					0.05	0.01
Time	3	13407.85	4469.29	117.37***	4.07	7.59
Treatments	1	2408.90	2408.90	63.26***	5.32	11.26
Interaction	3	2462.99	821.00	21.56***	4.07	7.59
ERROR	8	304.62	38.08			
TOTAL	15	18584.4				

APPENDIX 25a: Killing of carrot single cells by *Ecc* SCRI 1039 at 10^7, 10^8, 10^9 cfu/ml

ANALYSIS OF VARIANCE (Based on data from Chap. 3.III, Sec. 2.3., Figure 3.9a)

SOURCE	df	SS	MS	F	F_{table}	
					0.05	0.01
Time	7	24393.8	3484.8	69.7***	2.31	3.26
Treatments	3	2779.9	926.6	18.5***	2.9	4.46
Interaction	21	5570.3	265.3	5.3***	1.91	2.5
ERROR	32	1599.6	49.9			
TOTAL	63	34343.6				

APPENDIX 25b: Killing of carrot cell units by *Ecc* SCRI 1039 at 10^7, 10^8, 10^9 cfu/ml

ANALYSIS OF VARIANCE (Based on data from Chap. 3.III, Sec. 2.3., Figure 3.9b)

SOURCE	df	SS	MS	F	F_{table} 0.05	0.01
Time	7	43397.9	6199.7	120.9***	2.31	3.26
Treatments	3	10315.5	3438.5	67.1***	2.9	4.46
Interaction	21	14096.5	671.3	13.1***	1.91	2.5
ERROR	32	1640.6	51.3			
TOTAL	63	69450.6				

APPENDIX 26a: Determination of aggressiveness of *Ecc* on single cells viability at 10^3, 10^5, 10^7 cfu/ml

ANALYSIS OF VARIANCE (Based on data from Chap. 3.III, Sec. 2.4., Figure 3.10a)

SOURCE	df	SS	MS	F	F_{table} 0.05	0.01
Time	6	56306.3	9384.38	259.96***	2.45	3.53
Treatments	3	21068.4	7022.79	194.54***	2.95	4.57
Interaction	18	11157.8	619.88	17.17***	1.99	2.65
ERROR	28	1010.8	36.09			
TOTAL	55	89543.3				

APPENDIX 26b: Determination of aggressiveness of *Ecc* on cell units viability at 10^3, 10^5, 10^7 cfu/ml

ANALYSIS OF VARIANCE (Based on data from Chap. 3.III, Sec. 2.4., Figure 3.10b)

SOURCE	df	SS	MS	F	F_{table} 0.05	0.01
Time	6	56884.3	9480.72	302.17***	2.45	3.53
Treatments	3	20183.2	6727.72	214.43***	2.95	4.57
Interaction	18	10888.5	604.92	19.28***	1.99	2.65
ERROR	28	878.5	31.38			
TOTAL	55	88834.5				

APPENDIX 27: Growth of wild type (SCRI 193) and pectate lyase negative mutants (RJP 116, RJP 243) of *Ecc* in co-culture with carrot cv. Morot Duke suspension cells

ANALYSIS OF VARIANCE (Based on data from Chap. 3.III, Sec. 2.5., Figure 3.11)

SOURCE	df	SS	MS	F	F_{table}	
					0.05	0.01
Time	7	35677036	11892345	44.73***	2.77	4.16
Strains	2	12809087	6404543	24.09***	3.16	5.01
Treatments	1	23887872	23887872	89.85***	4.02	7.12
Time × Strains	6	16390101	2731683	10.28***	2.27	3.15
Time × Treatments	3	34909368	11636456	43.77***	2.77	4.16
Strains × Treatments	2	7378664	3689332	13.88***	3.16	5.01
ERROR	54	14356270	26585685			
TOTAL	71	145408400				

APPENDIX 28a: Killing of carrot single cells by *Ecc* mutants obtained from University of Warwick

ANALYSIS OF VARIANCE (Based on data from Chap. 3.III, Sec. 2.6.1.1., Figure 3.12)

SOURCE	df	SS	MS	F	F_{table}	
					0.05	0.01
Time	7	5460.28	780.04	43.45***	2.25	3.12
Strains	4	2594.08	648.52	36.12***	2.61	3.83
Interaction	28	3684.06	131.57	7.33***	1.76	2.23
ERROR	40	718.16	17.95			
TOTAL	79	12456.58				

APPENDIX 28b: Killing of carrot cell units by *Ecc* mutants obtained from University of Warwick

ANALYSIS OF VARIANCE (Based on data from Chap. 3.III, Sec. 2.6.1.1., Figure 3.12)

SOURCE	df	SS	MS	F	F_{table}	
					0.05	0.01
Time	7	25404.21	3629.17	112.94***	2.25	3.12
Strains	4	14352.84	3588.21	111.66***	2.61	3.83
Interaction	28	16313.06	582.61	18.13***	1.76	2.23
ERROR	40	1285.38	32.13			
TOTAL	79	57355.48				

APPENDIX 29a: Killing of carrot single cells by *Ecc* mutants obtained from University of Missouri-Colombia

ANALYSIS OF VARIANCE (Based on data from Chap. 3.III, Sec. 2.6.1.2., Figure 3.13)

SOURCE	df	SS	MS	F	F_{table} 0.05	0.01
Time	7	17785.9	2540.8	79.6***	2.21	3.04
Strains	5	6054.8	1210.9	37.9***	2.41	3.43
Interaction	35	4179.2	119.4	3.7***	1.67	2.06
ERROR	48	1531.4	31.9			
TOTAL	95	29551.3				

APPENDIX 29b: Killing of carrot cell units by *Ecc* mutants obtained from University of Missouri-Colombia

ANALYSIS OF VARIANCE (Based on data from Chap. 3.III, Sec. 2.6.1.2., Figure 3.13)

SOURCE	df	SS	MS	F	F_{table} 0.05	0.01
Time	7	51205.7	7315.1	485.8***	2.21	3.04
Strains	5	14705.3	2941.1	195.3***	2.41	3.43
Interaction	35	11859.6	338.8	22.5***	1.67	2.06
ERROR	48	722.8	15.6			
TOTAL	95	78493.4				

APPENDIX 30a: The effect of culture supernatants obtained from wild type SCRI 193 on suspension cultured single cells of carrot cv. Morot Duke

ANALYSIS OF VARIANCE (Based on data from Chap. 3.III, Sec. 2.7.1., Figure 3.14a)

SOURCE	df	SS	MS	F	F_{table} 0.05	0.01
Time	6	3655.05	609.18	44.83***	2.45	3.53
Treatments	3	16700.50	5566.82	409.69***	2.95	4.57
Interaction	18	3483.42	193.52	14.24***	1.99	2.65
ERROR	28	380.46	13.59			
TOTAL	55	24219.40				

APPENDIX 30b: The effect of culture supernatants obtained from wild type SCRI 193 on suspension cultured cell units of carrot cv. Morot Duke

ANALYSIS OF VARIANCE (Based on data from Chap. 3.III, Sec. 2.7.1., Figure 3.14b)

SOURCE	df	SS	MS	F	F_{table} 0.05	0.01
Time	6	18909.9	3151.6	320.4***	2.45	3.53
Treatments	3	84350.6	28116.9	2858.7***	2.95	4.57
Interaction	18	14343.6	796.9	81.0***	1.99	2.65
ERROR	28	275.4	9.8			
TOTAL	55	117879				

APPENDIX 31a: The effect of culture supernatants obtained from enzyme deficient mutant RJP 116 on suspension cultured single cells of carrot cv. Morot Duke

ANALYSIS OF VARIANCE (Based on data from Chap. 3.III, Sec. 2.7.2., Figure 3.15a)

SOURCE	df	SS	MS	F	F_{table} 0.05	0.01
Time	4	1894.99	473.75	23.15***	2.87	4.43
Treatments	3	810.98	270.33	13.21***	3.10	4.94
Interaction	12	552.17	46.01	2.25***	2.28	3.23
ERROR	20	409.23	20.46			
TOTAL	39	3667.38				

APPENDIX 31b: The effect of culture supernatants obtained from enzyme deficient mutant RJP 116 on suspension cultured cell units of carrot cv. Morot Duke

ANALYSIS OF VARIANCE (Based on data from Chap. 3.III, Sec. 2.7.2., Figure 3.15b)

SOURCE	df	SS	MS	F	F_{table} 0.05	0.01
Time	4	866.38	216.60	4.53***	2.87	4.43
Treatments	3	167.02	55.67	1.17***	3.10	4.94
Interaction	12	174.89	14.57	0.31***	2.28	3.23
ERROR	20	955.26	47.76			
TOTAL	39	2163.56				

APPENDIX 32: Effect of culture supernatants on suspension cultured cell units of carrot cv. Morot Duke

ANALYSIS OF VARIANCE (Based on data from Chap. 3.III, Sec. 2.8., Figure 3.16)

SOURCE	df	SS	MS	F	F_{table} 0.05	0.01
Time	7	6829.8	975.69	18.86***	2.21	3.04
Treatments	5	4654.4	9308.50	179.98***	2.41	3.43
Interaction	35	12229.5	349.41	6.76***	1.67	2.11
ERROR	48	2482.6	51.72			
TOTAL	95	68084.3				

APPENDIX 33a: The effect of antibiotics on suspension cultured single cells of carrot cv. Morot Duke

ANALYSIS OF VARIANCE (Based on data from Chap. 3.III, Sec. 2.9.2., Figure 3.17)

SOURCE	df	SS	MS	F	F_{table} 0.05	0.01
Time	6	17739	2956.50	146.96***	2.22	3.05
Treatments	10	51452.4	5145.24	255.76***	1.96	2.56
Interaction	60	13105.7	218.43	10.86***	1.49	1.76
ERROR	77	1549.1	20.12			
TOTAL	153	83846.2				

APPENDIX 33b: The effect of antibiotics on suspension cultured cell units of carrot cv. Morot Duke

ANALYSIS OF VARIANCE (Based on data from Chap. 3.III, Sec. 2.9.2., Figure 3.17)

SOURCE	df	SS	MS	F	F_{table} 0.05	0.01
Time	6	109088	18181.37	514.94***	2.22	3.05
Treatments	10	63565.6	6356.56	180.03***	1.96	2.56
Interaction	60	25703.5	428.39	12.13***	1.49	1.76
ERROR	77	2718.7	35.31			
TOTAL	153	201076				

APPENDIX 34a: Single cells viability of carrot cv. Morot Duke suspension culture inoculated with *P. violae*

ANALYSIS OF VARIANCE (Based on data from Chap. 3.III, Sec. 3.1., Figure 3.18)

SOURCE	df	SS	MS	F	F_{table} 0.05	F_{table} 0.01
Time	3	7979.28	2659.76	304.62***	3.24	5.29
Treatments	3	1655.95	551.99	63.22***	3.24	5.29
Interaction	9	2833.32	314.81	36.05***	2.54	3.78
ERROR	16	139.71	8.73			
TOTAL	31	12608.3				

APPENDIX 34b: Cell units viability of carrot cv. Morot Duke suspension culture inoculated with *P. violae*

ANALYSIS OF VARIANCE (Based on data from Chap. 3.III, Sec. 3.1., Figure 3.18)

SOURCE	df	SS	MS	F	F_{table} 0.05	F_{table} 0.01
Time	3	26494.2	8831.39	70.92***	3.24	5.29
Treatments	3	16479.7	5493.23	44.11***	3.24	5.29
Interaction	9	11738.2	1304.25	10.47***	2.54	3.78
ERROR	16	1992.51	124.53			
TOTAL	31	56704.6				

Appendix 35: Composition of Murashige and Skoog (1962) and modified Lin and Staba basal medium (1979)

	Media	
COMPONENTS	**MS (1962) (mg/l)**	**Modified Lin and Staba (1979) (mg/l)**
NH_4NO_3	1650	267.5
KNO_3	1900	5550
$CaCl_2.2H_2O$	440	219.9
$MgSO_4.7H_2O$	370	185
KH_2PO_4	170	68
Na_2-EDTA	37.3	37.3
$FeSO_4.7H_2O$	27.8	27.8
H_3BO_3	6.2	2.4
$MnSO_4.4H_20$	22.3	9.24
$ZnSO_4.7H_20$	8.6[a]	4.05
KI	0.83	0.375
$Na_2MoO_4.2H_2O$	0.25	0
$CuSO_4.5H_2O$	0.025	0
$CoCl_2.6H_2O$	0.025	0
myo-Inositol	100	0
Nicotinic acid	0.5	5
Pyridoxine HCl	0.5	0.5
Thiamine HCl	0.1	3
Sucrose	3%	2%

[a] : $ZnSO_4.4H_20$

II. POSTER PRESENTATION

Poster presented at VIIIth International Congress of Plant Tissue and Cell Culture Firenze, Italy. June 12-17, 1994.

ABSTRACT

Embryogenic callus and suspension cultures of carrot *(Daucus carota L.)* have been developed in order to generate, via somaclonal variation, disease resistance to three major pathogens of carrot in Europe: *Erysiphe heraclei* (powdery mildew), *Pythium violae* (cavity spot) and *Erwinia carotovora subsp. carotovora* (soft rot). Some of somaclones produced from callus have shown resistance to *E. heraclei* and investigations are underway to confirm this observation and to determine its heritability. Co-culture of plant cells and *E. carotovora* has been attempted with the possible aim of regenerating plants from surviving cells. Cells however, were killed very rapidly (some within 2.5 hrs) and attempts are now being made to reveal the cell killing factor(s) from *E. carotovora* especially by employing defined mutants with defects in, for example secretion or production of cell wall-degrading enzymes. In some mutants cell killing, occurred in the absence of pectate lyase, generally considered as the most obvious candidate for a toxic role. These mutants may be of value in screening carrot cells for disease resistance *in vitro*.

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